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Direttore responsabile: Prof. Edoardo Ascari; Autorizzazione del Tribunale di Pavia n. 63 del 5 marzo 1955.
Editing: Mikimos - Medical Editions, via A. Fogazzaro 5, Voghera, Italy
Printing: Tipografia PI-ME, via Vigentina 136, Pavia, Italy
Printed in September 2004

Haematologica is sponsored by educational grants from the following institutions and companies:

IRCCS Policlinico S. Matteo, Pavia, Italy
University of Pavia, Italy
José Carreras International Leukemia Foundation
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The origin and power of a name

Ancient Greek

αἷµα [aima] = blood;
αἷµατος [aimatos] = of blood,
λόγος [logos] = reasoning

Scientific Latin

haematologicus (adjective) = related to blood

Scientific Latin

haematologica (adjective, plural and neuter, used as a noun) = hematological subjects

Modern English

Journal of Hematology
2003 JCR® Impact Factor = 3.453
# VIII Congress of the Italian Society of Experimental Hematology

*Pavia, September 14-16, 2004*

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A great effort is being spent to develop noninvasive diagnostic procedures based on the analysis of fetal genetic material obtained from the maternal circulation. Recent studies showed the presence of free fetal DNA and RNA circulating in plasma of pregnant women. The mechanisms underlying fetal DNA release and clearance into and from maternal circulation are still unclear, despite some hypotheses have been postulated. The main sources which have been advocated for this phenomenon are lysis of damaged fetal cells by the maternal immune system, or programmed cell death following developmental remodeling of certain fetal tissues. Aim of our work is to investigate physiopathological factors which may influence fetal nucleic acid concentration in maternal plasma and develop a model assay for noninvasive prenatal diagnosis of genetic disorders. To this purpose, we evaluated on a large cohort of physiological pregnancies whether fetal DNA can be retrieved at any gestational week at sufficient quantity to be analyzed with advanced mutation detection technologies. Fetal DNA quantification was carried out through real-time PCR by amplifying Y-specific sequences in male-bearing pregnancies as the simplest way to distinguish between maternal and fetal DNA. Fetal DNA was detected in 277 women with previous sons or abortions. These findings confirm that maternal plasma may represent the optimal source of fetal genetic material. We also evaluated fetal DNA concentration in pregnancies complicated by placental pathologies. In our cohort of IUGR pregnancies a highly statistically significant fetal DNA increase in maternal plasma was found compared to normal controls (median 155 ge/mL), close to the more than fourfold increase found in our preeclampsia cohort (median 195.2 ge/mL). Remarkably, we also found that the association between preeclampsia and IUGR further increases up to sevenfold these values (median 394.1 ge/mL), suggesting a consistent independent contribution of IUGR to this phenomenon. We are also exploring the feasibility of fetal RNA analysis in maternal plasma in order to evaluate whether this may be a better early predictive marker of the development of feto-placental pathologies than fetal DNA. To this aim we will continue to perform quantitative analysis of both DNA and RNA in maternal plasma collected from pregnancies affected or at risk of developing preeclampsia and/or IUGR. For noninvasive diagnosis of genetic diseases we are presently evaluating a microchips technology. We set up assays to detect 9 beta-thalassemia mutations. The detection limit for a mutated genomic DNA sample diluted into a wild-type DNA sample was 5 genome equivalents suggesting that this method has the potential for noninvasive diagnosis of fetal mutations in maternal plasma.

Funded by Telethon GGP02015 and MIUR Cofin 2002.

GENETIC DISORDERS OF IRON METABOLISM AS A MODEL OF GENOMIC MEDICINE
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Recent advances in the molecular genetics of hereditary hemochromatosis have elucidated the pathogenesis of the disease, which involves the
hepatic peptide hepcidin, a key regulator of iron metabolism in mouse models. In normal conditions only 1-2 mg iron are absorbed daily from the gut and 25-30 mg are released from macrophages to circulating transferrin. These amounts can be increased according to the needs of the erythron (erythroid regulator) or reduced on the basis of iron stores (store regulator). In Hemochromatosis iron absorption and release are greater than normal and are unrelated to the body needs, making this disorder a suitable model to study iron homeostasis. Different genetic types of Hemochromatosis have been identified. HFE-related Hemochromatosis, the first recognized disorder, affects prevalently middle aged males, has low penetrance and variable clinical phenotype. Juvenile Hemochromatosis on the contrary is characterized by early onset, severe iron overload and clinical complications in both sexes. Iron absorption in HFE Hemochromatosis can still increase, whereas it is maximal in the juvenile type. The present evidence indicates that both disorders involve hepcidin. Juvenile Hemochromatosis results from the absence of hepcidin, rarely through direct mechanisms (hepcidin mutations), more commonly through mutations of the newly discovered hemojuvelin protein. Patients with hemojuvelin mutations have no measurable urinary hepcidin. Evidence is accumulating that HFE-Hemochromatosis is due to reduced hepcidin production, which is inappropriate to the degree of iron loading. Accordingly, HFE is involved in the hepcidin increase that follows iron loading and it is thus a component of the store regulator, whose function is to block iron absorption. The Juvenile Hemochromatosis proteins, hepcidin and hemojuvelin, are components of the erythroid regulator, which requires that hepcidin is switched off to allow maximal iron absorption. A third type of Hemochromatosis is due to inactivation of transferrin receptor 2 (TFR2), a member of the transferrin receptor family, whose function in iron metabolism is uncertain. The resulting disorder is characterized by iron overload early in life, but runs a clinical course milder than the juvenile form. The TFR2 relationship with hepcidin is still speculative. From clinical and experimental findings we suggest that TFR2 has a role distinct from HFE in hepcidin activation. This is in agreement with the observation that normal TFR2 cannot compensate the lack of HFE in HFE-Hemochromatosis. Genomic medicine applied to Hemochromatosis is opening new perspectives in our understanding iron homeostasis.

REGULATION OF ERYTHROPOIESIS
Vannucchi AM
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Erythropoiesis, the events that lead to the formation of mature erythrocytes, is the result of complex differentiation processes that, starting from the primitive hematopoietic stem cell, proceed through a number of intermediate progenitor and precursors cells. The cellular compartments involved in this process have been identified and characterized based on their ability to give rise to colonies of different appearance (CFU-GEMM, BFU-E, CFU-E) and proliferative potentialities when cultured in vitro under defined stimulatory conditions; one of the essential events in the process is the expression on the membrane of developing cells of receptors for erythropoietin (EPO), whose density reaches a maximum of around 1,000 molecules/cells at the proerythroblast stage. The binding of EPO to the receptor homodimer induces a change in the conformational status of the molecule with ensuing activation of the Janus kinase 2 (JAK2), that on turn binds EPOR itself at the level of a Box1 motif. Downstream signalling pathways involve at least phosphatidylinositol 3 kinase, Stat5, and Ras. The essential non-redundant role of EPO and its receptor in erythropoiesis has been strengthened by the generation of mice with deficiency of the corresponding gene (knockout mice). The liver of both Epo−/− and EpoR−/− fetus contains normal numbers of BFU-E and CFU-E progenitors, suggesting that neither the hormone nor the receptor are required for erythroid lineage commitment of the hematopoietic stem cell; however, definitive erythropoiesis is blocked, resulting in embryonic mortality due to severe anemia. Indeed, the principal function of EPO-EPOR system is that of rescuing committed progenitors from apoptosis, at least partially by regulating the expression of bcl-xL. A similar phenotype has been shown also for Stat5a−/−5b−/− embryos, that are severely anemic due to a high rate of progenitor apoptosis as the consequence of defective activation of the bcl-xL promoter by Stat5. Of note, the defect induced by the Stat5a−/−5b−/− genetic manipulation is not apparent in the adult life due to compensatory mechanisms possibly involving Stat1 and/or Stat3. Among other cytokines playing a role in erythropoiesis, the kit ligand (KL, or stem cell factor, SCL) and its receptor (c-kit, or SCFR) are preferentially involved in the early stages of progenitor proliferation and maturation; the number of SCFR molecules progressively declines from pluripotent hematopoietic progenitors to CFU-E, and are absent in the morphologically recognizable erythroblasts. Co-administration of SCF and EPO, both in vitro and
in vivo, results in potent stimulation of erythropoiesis. Finally, non-cytokine mediated signals may have a profound impact on erythropoiesis, as it is the case for glucocorticoids, whose stimulatory effects are supported by a number of clinical and experimental observations; they might be especially involved in the regulation of stress erythropoiesis, since mice genetically deficient in glucocorticoid receptors mount an inappropriate erythroid response. The activity of glucocorticoids in stress erythropoiesis is antagonized by p53, as shown by the faster response to anemia in p53+/− mice; one mechanism for this interaction might be the ability of p53 to inhibit the expression of c-myb, therefore favouring differentiation versus proliferation. The existence of strict connections between the erythroid and the megakaryocytic differentiation lineages, initially supposed from the frequent coexpression of erythroid and megakaryocytic phenotypic markers and differentiation potentials in human and erythroleukemic cell lines, has received experimental validation in the last few years following the demonstration that: i) a common bipotent erythroid-megakaryocytic progenitor (MEP) normally exists in hematopoiesis; ii) during normal and, especially, under stress erythropoiesis in the mouse, bipotent, non-clonogenic erythroid-megakaryocytic progenitors are found in both the bone marrow and the spleen; and iii), by manipulating in vitro culture conditions, mature glycophorin-A+ erythroblasts retain the potentiality to differentiate into CD41+ polyploid megakaryocytes.

The different processes that occur during erythropoiesis are regulated at the molecular level by a network of interacting transcription factors, that ultimately act by regulating the transcriptional activity of erythroid-specific genes, at the same time exerting complex self-regulatory functions. The erythroid-specific prototype transcription factor is represented by GATA-1, a zinc-finger protein whose consensus sequences have been found in the promoters and/or enhancers of all erythroid genes examined up to now (including globin genes, EPOR, and GATA-1 itself); the gene for GATA-1 is on the X-chromosome. Gata-1 is first activated at the level of pluripotent hematopoietic progenitors, then its expression is either down-regulated during myeloid commitment, or maintained and progressively increased in progenitors committed to the erythroid lineage. GATA-1 molecules contain a COOH-terminal and N-terminal zinc finger, and a region involved in the stabilization of binding to palindromic DNA sequences. The amino zinc finger is required for the binding of an obligatory cofactor, named FOG-1 (that stands for Friend Of GATA-1), that is strictly required for the functional activity of GATA-1 on target genes. Beyond the erythroid one, Gata-1 is expressed in cells of megakaryocytic, mast cell, and eosinophilic lineages, and also in Sertoli cells of the developing testis in the mouse. The essential role of GATA-1 in normal erythropoiesis has been shown by gene targeting studies in mice, that lead to the generation of several mutants either knockout or knockdown for the gene. Hemizygous male Gata-1 knockout mice die early in gestation due to severe anemia with arrest of maturation at a proerythroblast stage. GATA-1−/− cells undergo rapid apoptosis, indicating an essential role of GATA-1 in both cell survival and cell maturation. In vitro, GATA-1, also in cooperation with EPO, selectively induces the expression of bcl–xL but not of bcl-2, maintaining erythroid cell viability during terminal maturation. Cellular levels of GATA-1 at 1/4 of normal ones, such as in Gata-1low knockdown mice, are relatively well tolerated in the adult life, probably due to some still unclear compensatory mechanism; furthermore, in some experimental conditions, other transcription factors, such as GATA-2, may substitute for GATA-1. On the other hand, the erythroid phenotype of FOG-1−/− mice was reminiscent that of Gata-1 knockouts, while, interestingly, these mice showed a complete defect of megakaryocytopoiesis to suggest a GATA-1 independent role in early steps of megakaryocytopoiesis. Other transcription factors that serve a role in erythropoiesis are represented by EKLF, NF-E2, and SCL/tal1; about the latter, conditional knockout of SCL/tal1 showed reduced basal and stress erythropoiesis due to the loss of early progenitors, a defect in common with the megakaryocytic lineage, while myeloid cell development was unaffected.

Studies of transcriptional profile and activity of different classes of erythroid progenitor and precursor cells, together with the invaluable in-vivo models represented by genetically modified animals, are providing new information about the regulation of erythropoiesis not only under steady state, but especially under stress conditions; hopefully, these information will help in the understanding of the pathogenesis of complex anemias, and possible, in the development of more specific therapies.

THE MOLECULAR REGULATION OF MEGAKARYOCYTE DIFFERENTIATION

Catani L
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Cells comprising the megakaryocyte (MK) lineage include a bipotential erythro–MK progenitors, MK progenitors (BFU-MK and CFU-MK), post-mitotic MKs still capable of undergoing endoreduplication and more differentiated polyploid MKs undergoing terminal maturation. This process is regulated by a
complex network of interacting cytokines, growth factors and extracellular matrix proteins. The exact nature of master control genes regulating MK lineage-specific development remains still obscure. However, genetic studies have recently provided rich insights into the molecular and transcriptional regulation of MK differentiation and thrombopoiesis. Furthermore, knowledge accumulated by studying mouse models and the defects in patients with hereditary thrombocytopenia. Recent advances have highlighted the particular importance of four transcriptional regulators, GATA-1, FOG-1, FLI-1, and NF-E2, of distinct stages in MK differentiation, extending from the birth of early committed progenitors to the final step of platelet release. Studies involving selective knockout of GATA-1 expression in Mk of mice and others involving overexpression of GATA-1 in cell line models revealed a key role for GATA-1 in the differentiation of MKs. Most recently, GATA-1 mutations altering either the FOG-binding or the DNA binding domain of the protein have been described to be associated with human inherited diseases characterized by alterations in the MK and the erythroid differentiation pathways. NF-E2 is a hetero-dimeric factor and the p45 subunit appears to be free to associate with any of the three small-Maf subfamily. Mice lacking p45 NF-E2 show megakaryocytosis and severe thrombocytopenia leading to fatal hemorrhage. Loss of p45 NF-E2 interferes with MK maturation at an advanced stage: ultrastructural studies reveal a markedly reduced number of granules and failure to form platelet fields. Therefore genes that are regulated by NF-E2 are critical to terminal Mk maturation and platelet formation. However, up to date, no human diseases have been associated with NF-E2 deregulation. Experimental evidences suggest also that the FLI-1 oncogene, associated with Ewing’s sarcoma in humans and experimental Friend virus-induced erythroleukemia in mice, plays an important role during the normal development of MKs. Inactivation of the FLI-1 gene in mice is embryonic lethal. FLI-1 knockout mice produce small, undifferentiated MK progenitors with abnormal ultrastructural features such as reduced alpha-granules numbers and disrupted demarcation membrane systems. Levels of MK-specific genes normally expressed late during differentiation, such as GPIX, are also markedly reduced. An especially rare condition called Paris-Trousseau syndrome seems to occur by virtue of hemizygous loss of the FLI-1 gene. On this basis, within a linear hierarchy of transcriptional regulation in MK differentiation, the transcriptional control of the genesis of the bi-potential erythro-MK progenitors remains mysterious. Experimental evidences suggest that FOG-1, a GATA-1 co-activator, acts early in MK development without the involvement of GATA-1 and then again later in a GATA-dependent pathway. Studies on mouse and human MK colonies point to positive regulation of proliferation of MK progenitors by NF-E2 and negative regulation by GATA-1. Further cytoplasmic maturation is impaired in the absence of FLI-1 or GATA-1 in mice and when human GATA-1 is mutated such that it interacts poorly with its cofactor FOG-1. Finally, platelet release is regulated by NF-E2, probably through coordinate activation of many genes that reorganize the cytoskeleton and transport organelles into proplatelets.

References

TRANSCRIPTIONAL TARGETS OF ACUTE MYELOID LEUKEMIA FUSION PROTEINS

Alcalay M

IPIOM – Fondazione Istituto FIRC di Oncologia Molecolare, Milan; IEO – Istituto Europeo di Oncologia, Milan, Italy

Acute myelogenous leukemias (AMLs) are characterized by heterogeneous chromosomal rearrangements that generate fusion proteins with aberrant transcriptional regulatory activities. AML fusion proteins interfere with the process of myeloid differentiation, determine a stage-specific arrest of maturation and enhance cell survival, and transgenic mice display increased risk of myeloid leukemias,

haematologica vol. 89(suppl. n. 6):september 2004
suggeting that they induce a pre-leukemic state. 1-3 The abnormal regulation of transcriptional networks induced by AML-fusion proteins occurs through common mechanisms that include recruitment of aberrant corepressor complexes, alterations in chromatin remodeling, and disruption of specific subnuclear compartments. 4,5 The identification and analysis of common and specific target genes regulated by AML fusion proteins is of fundamental importance for the full understanding of acute myeloid leukemogenesis and for the implementation of disease-specific drug design. We have performed a systematic analysis of fusion protein targets genes in diverse model systems using Affymetrix oligonucleotide chips. First, we analyzed transcriptional modulation after expression of the AML1/ETO, PML/RAR and PLZF/RAR fusion proteins in U937 hemopoietic precursor cells. 6 We thus identified 1555 genes regulated concordantly by at least two fusion proteins that were further validated in patient samples, and finally classified according to available functional information. The analysis of functionally homogenous groups of genes that are coherently regulated by fusion proteins revealed that: i) genes involved in in maintenance of the stem-cell phenotype are induced; ii) genes that regulate hematopoietic stem cell (HSC) commitment or differentiation are repressed, iii) DNA repair genes, mainly of the base excision repair (BER) pathway, are repressed. Functional studies confirmed that ectopic expression of AML fusion proteins constitutively activates pathways leading to increased stem cell renewal (e.g. the Jagged1/Notch pathway) and provokes accumulation of DNA damage through repression of BER. Expansion of the stem cell compartment and induction of a mutator phenotype may therefore represent relevant features underlying the leukemic potential of AML-associated fusion proteins. Acute promyelocytic leukemia (APL) is a distinct subtype of AML characterized by a differentiation block at the promyelocytic stage. APL patients respond to pharmacological concentrations of all-trans retinoic acid (RA) and, at the cellular level, disease remission correlates with terminal differentiation of leukemic blasts. 7,8 The PML/RAR oncogenic transcription factor is responsible for both the pathogenesis of APL and for its sensitivity to RA. 9 PML/RAR functions by de-regulating RA target genes critical to myeloid differentiation, which are thought to represent the downstream effectors of the fusion protein’s oncogenic potential. RA is thought to act by antagonizing PML/RAR-dependent gene regulation, thereby favoring terminal differentiation. Analysis of the regulatory pathways impaired during leukemogenesis and reactivated during RA-induced differentiation may therefore contribute to the identification of new molecular targets for leukemia therapy. We analyzed gene expression profiles of RA-treated APL blasts with the aim of identifying physiological targets of RA therapy, and identified 1056 common target genes. 9 We compared these results to those obtained upon RA treatment of U937 cells lines, and found that transcriptional response to RA is largely dependent on the expression of PML/RAR. Several genes involved in the control of differentiation and stem cell renewal are early targets of RA regulation, and may be direct effectors of RA response. Modulation of chromatin modifying genes was also observed, suggesting that specific structural changes in local chromatin domains may be required to promote RA-mediated differentiation. Mutual cross-talk between transcriptional regulatory pathways is essential for triggering differentiation. Computational analysis of upstream genomic regions in RA target genes revealed non-random distribution of transcription factor binding sites, indicating that specific transcriptional regulatory complexes may be involved in determining RA response.

References


STEM CELLS MOBILIZATION

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Cytokine-mobilized hematopoietic stem cells (HSCs) have significantly increased indications and feasibility of stem cell transplantation (SCT), while dramatically reducing transplant-related morbidity and mortality. Autologous HSCs have now an estab-
lished role in the management of neoplastic diseases, such as non-Hodgkin lymphomas or acute myelogenous leukemias, and their use is also increasingly explored in a variety of cancers who might benefit of dose-intensification. Similarly, allogeneic HSCs represent the preferred stem cell source in HLA-mismatched transplantation and are gaining an increased consensus for HLA-identical allografting. The availability of adequate amounts of HSCs represents an essential prerequisite for the feasibility of high-dose therapy programs. Optimal mobilization of autologous peripheral blood progenitor cells (PBPCs) in cancer patients requires both chemotherapy and growth factors, whereas allogeneic PBPCs are mobilized by short courses of granulocyte colony-stimulating factor (rhG-CSF). Due to either prior chemotherapy or disease-related factors, a substantial proportion of cancer patients fail to mobilize optimal amounts of CD34+ cells. Failure to mobilize the required target cell dose of CD34+ cells may also occur in healthy donors. Improving stem cell mobilization is of relevance not only for poor mobilizers or graft engineering procedures, but also for gene therapy programs as well as for stem cell–based cell-replacement therapy. Stem cell mobilization might be enhanced either (i) by interfering with the mechanism(s) regulating hematopoietic stem cell trafficking, i.e., transmigration through the luminal endothelium to extravascular bone marrow spaces in homing and the reverse in mobilization, or (ii) by using early-acting cytokines capable of expanding marrow progenitors.

**Drugs interfering with stem cell trafficking.** The localization of hematopoietic cells to the bone marrow involves developmentally regulated adhesive interactions between hematopoietic cells and stromal cells. A wide variety of cell adhesion molecules (CAMs) participate in the adhesion of HSCs to stromal cells and their associated extracellular matrix components. The α4β1 integrin plays a key role in homing of HSCs/HPCs to marrow stroma and administration of function-blocking antibodies against α4β1 integrin or its cellular receptor, vascular cell adhesion molecule-1 (VCAM-1), inhibits homing and induces PBPC mobilization.

The polydeoxyribonucleotide Defibrotide is avidly bound to vascular endothelium and significantly decreases expression of CAMs, such as P-selectin and intercellular adhesion molecule-1 (ICAM-1), on endothelial cells. We have recently shown that in mice and nonhuman primates, Defibrotide significantly enhances the frequency and absolute number of a broad spectrum of hematopoietic progenitors, including committed progenitors and primitive LTC-IC, which are mobilized into the circulation by rhG-CSF. In mice, on day 3 of treatment, the combined Defibrotide/rhG-CSF injection results in WBC and PBPC counts equal to those observed after 5 days of rhG-CSF injection, suggesting that Defibrotide addition may decrease the frequency of administration and total amount of rhG-CSF required for harvesting sufficient blood stem cells for transplantation. Alternatively, the combined Defibrotide/rhG-CSF mobilization regimen may significantly increase the total dose of PBPCs collected during a standard mobilization procedure in healthy donors or cancer patients. In vivo homing experiments in mice show that Defibrotide administration reduces bone marrow homing of transplanted CFCs or CFDA-SE-stained Sca-1+lin− cells while inducing their accumulation in the blood, strongly suggesting that Defibrotide-enhanced mobilization is mediated by disruption of the recirculation of mobilized progenitors back into bone marrow, and not by an additive effect on the physiology of rhG-CSF administration.

**Drugs expanding bone marrow progenitors.** Human growth hormone (rhGH) has been shown to have a remarkable promoting activity on the in vitro growth of human erythroid and myeloid progenitors. In vivo, rhGH acts as a multilineage hematopoietic growth factor. In mice, rhGH given for 7 days increases the number of splenic and bone marrow progenitor cells, as well as bone marrow cellularity. In rats, rhGH given for 28 days reverses age-associated loss of bone marrow hematopoietic cells. By studying mice with impaired bone marrow function due to aging and irradiation, we have recently shown that injection of rhGH (2.5 mg/kg/day, i.p., for 35 days) is associated with: (i) reduction in the number of adipocytes and increase in the number of marrow hematopoietic cells; (ii) significant increase (p ≤ 0.05) of the mean femur CFC content with growth values approaching those of young control animals; (iii) restoration of rh-G-CSF-elicited mobilization of PBPCs. Based on these pre-clinical studies, the activity of rhGH in enhancing CD34+ cell mobilization elicited by chemotherapy plus rhG-CSF has been evaluated in 16 hard-to-mobilize patients, i.e., those achieving a peak of circulating CD34+ cells ≤10/µL, or a collection of CD34+ cells ≤2x10^6/kg. Patients who had failed a first mobilization attempt with chemotherapy plus rhG-CSF (5 µg/kg/day) were re-mobilized with chemotherapy plus rhG-CSF and rhGH (100 µg/kg/day). As compared with rhG-CSF, the combined rhGH/rhG-CSF treatment induced significantly higher (p ≤ 0.05) median peak values for CD34+ cells/µL (7 vs 29), CFCs/ml (2,154 vs 28,510), and LTC-IC/ml (25 vs 511). Following rh-G-CSF and rhGH/rhG-CSF, the median yields of CD34+ cells per leukapheresis were 1.1x10^6/kg and 2.3x10^6/kg (p ≤ 0.008), respectively; the median total collection of CD34+ cells were 1.1x10^6/kg and 6x10^6/kg (p=0.008), respectively. No specific side effect could be ascribed to rhGH, except a transient hyperglycemia occurring in two patients. Reinfusion
of rhGH/rh-CSF-mobilized cells following myeloabative therapy resulted in prompt hematopoietic recovery. In conclusion, our data demonstrate that in poor mobilizers addition of rhGH to rhG-CSF allows to efficiently mobilize and collect CD34+ cells with maintained functional properties. In conclusion, drugs interfering with stem cell trafficking or cytokines capable of expanding bone marrow progenitors are now available and represent attractive tools to be explored in the setting of stem cell mobilization.

References


ROLE OF NK CELL ALLOREACTIVITY IN ALLOGENIC HEMATOPOIETIC TRANSPLANTATION FOR ACUTE MYELOID LEUKEMIA

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HLA haplotype-mismatched transplants are at risk of T cell mediated allogeneic reactions in the host-versus-graft as well as in the graft-versus-host direction. Rejection is controlled by the conditioning regimen and "megadoses" of hematopoietic cells. GVHD is prevented by T cell depletion of the graft. These transplants however can rely on another type of alloreactivity, mediated by natural killer (NK) cells, which is triggered by MHC mismatches between specific NK clones in the donor repertoire and their inhibitory HLA class I ligands on recipient cells. HC molecules protect normal cells from NK cell-mediated lysis. The lack of expression of self MHC molecules on target cells results in suspetibility to NK cell-mediated lysis (missing self recognition). Human NK cells discriminate between allelic forms of MHC molecules via clonally-distributed receptors, termed KIRs (from Killer Cell Ig-like Receptor), that are specific for epitopes that are shared by MHC class I alleles, i.e., group 1 and group 2 HLA-C alleles, and HLA-Bw4 allele (Table 1). As KIRs are clonally distributed, each cell in the repertoire bears a different receptor or, less frequently, two or more receptors. However, every mature NK cell expresses at least one receptor that is specific for self HLA class I molecules. This ensures the generation of alloreactive NK cells between individuals who are mismatched for either one of the two subgroups of HLA-C alleles and/or the HLA-Bw4 allele group. For example, individuals who express Group 2 HLA-C alleles and possess NK cells that express KIR specific for Group 2 HLA-C alleles (KIR2DL1) are alloreactive against cells from individuals who do not express Group 2 HLA-C alleles (who are homozygous for Group 1 HLA-C alleles). Individuals who express Group 1 HLA-C alleles possess NK cells with KIR specific for Group 1 HLA-C alleles (KIR2DL2 and/or KIR2DL3) and are alloreactive against cells from individuals who do not express Group 1 HLA-C alleles (who are homozygous for Group 2 HLA-C alleles) (Figure 1). In HLA haplotype-mismatched hematopoietic transplantation with a potential for GVH NK-mediated reactions, the engrafted stem cells give rise to an NK cell wave of donor origin which regenerates the same repertoire as the donor’s, and so includes high-frequencies of donor-vs-recipient alloreactive NK cells. Donor-versus-recipient NK cell alloreactivity reduced the risk of leukemia relapse in 57 acute myeloid leukemia (AML) patients at high risk of relapse, while improving engraftment and protecting against GVHD.

<table>
<thead>
<tr>
<th>KIR</th>
<th>HLA class I specificity</th>
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<tbody>
<tr>
<td>KIR2DL1</td>
<td>“Group 2” HLA-C alleles expressing Cw1, Cw3, Cw4, Cw5, Cw6</td>
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<tr>
<td>KIR2DL2</td>
<td>“Group 1” HLA-C alleles expressing Cw1, Cw2, Cw3, Cw4, Cw5</td>
</tr>
<tr>
<td>KIR2DL3</td>
<td>HLA-Bw4 allele (e.g., HLA-B27)</td>
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Table 1. HLA class I allele specificity of the main inhibitory KIR.
An updated analysis of transplantation outcomes of 93 AML patients transplanted from haploidentical donors from 1993 through January 2004 demonstrates transplantation from NK alloreactive donors enhances engraftment (reaction rate 10% in the absence of NK alloreactivity, vs 2% in its presence), does not increase the risk of GvHD, but indeed appears to protect from it (9% vs 3%) and exerts a remarkable control of leukemia relapse. Probability of relapse is 68% for the 53 patients transplanted from non-NK alloreactive donors vs 15% (p<0.005) for the 40 patients transplanted from NK alloreactive donors. Probability of event free survival is 55% for patients with NK alloreactive donors vs 12% for those without (p<0.005). Thus, transplantation from an NK alloreactive donor is a strong independent factor predicting survival (transplantation from NK alloreactive vs non-NK alloreactive donor: hazard ratio = 0.44, C. I. = 0.25-0.77, p=0.004), even when compared with disease status at transplant (remission vs relapse: hazard ratio = 0.47, C. I. =0.28-0.73, p=0.004).

Figure 1. Donor-vs-recipient NK cell alloreactivity. Individuals who express Group 2 HLA-C alleles and possess NK cells that express KIR specific for Group 2 HLA-C alleles (KIR2DL2) are alloreactive against cells from individuals who do not express Group 2 HLA-C alleles (who are homozygous for Group 1 HLA-C alleles) (top). Individuals who express Group 1 HLA-C alleles possess NK cells with KIR specific for Group 1 HLA-C alleles (KIR2DL2 and/or KIR2DL3) and are alloreactive against cells from individuals who do not express Group 1 HLA-C alleles (who are homozygous for Group 2 HLA-C alleles) (bottom).

STRATEGIES TO MODULATE GRAFT-VS-HOST DISEASE

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Graft versus leukaemia (GvL) reactions constitute the major therapeutic component of allogeneic stem cell transplantation (SCT), and are responsible for eradicating the residual malignancy. This function has been largely attributed to donor T cells, because of the increased incidence of leukaemia relapse with T cell depleted inoculums of stem cells. The GvL effect has been associated with an expansion of minor histocompatibility (H) specific T cells which also exhibit a suppressive effect on the growth of leukemic precursors. 1 Furthermore, the adoptive transfer of minor H antigen specific T cells has recently been investigated as a treatment for non haemopoietic malignancies. 2 Unfortunately many minor antigens

References
are ubiquitously expressed, thus T cells may also target normal tissues leading to graft versus host disease (GvHD). Minor H antigen specific T cells are in fact detectable in patients with active GvHD. In agreement with these findings, we have found that pregnancy is able to prime minor H antigen specific T cell responses, both autosomal and anti HY (male specific), thus explaining the increased incidence of clinical GvHD when multiparous female are used as stem cells. Maximising GvL responses while minimising any accompanying GvHD presents a major challenge to the more extensive application of these therapies. A strategy adopted in the attempt to separate the GvL and GvHD effects, is to target minor H antigens expressed specifically on haemopoietic tissues such as HA-1 and HA-2. In a skin explant assay, T cells specific for the ubiquitously expressed HY antigen were able induce high grade GvHD, whereas haemopoietic specific T cells only induced GvHD if skins were previously incubated with HA-1 and HA-2 peptides. Other groups have observed that the infusion of T cells specific for a single immunodominant minor H antigen, irrespective of its tissue specific expression, can eradicate leukaemic cells without causing GvHD. Our clinical data with the use of donor lymphocyte infusions (DLI) to treat leukaemia relapse after allografting shows that the timing of DLI influences the occurrence and severity of GvHD. The incidence of GvHD in patients receiving DLI more than one year post transplant compared favourably with that of those who received DLI within the first year (14% and 46%, respectively). In fact, pre-transplant conditioning causes changes to gut epithelium, resulting in systemic dissemination of bacterial endotoxins, thereby initiating a cytokine cascade (cytokine storm) producing an inflammatory environment favouring GvHD. We have demonstrated that the in vitro migration of T cells in the presence of irradiated endothelial cells is greatly enhanced. We have confirmed these findings have been confirmed in vivo in animal models. An alternative approach to reduce GvHD rely on the exploitation of CD4+CD25+ immunoregulatory T cells (Treg). A few studies in murine models have documented that Treg prevents acute GvHD. Interestingly this GvHD modulation by donor Treg seems to occur with preservation of the graft versus tumor effect (GvT) in mice. We have observed that patients who relapse after allografting exhibit a high number of circulating CD4+CD25+ Treg. This increase is paralleled by an enhanced immunosuppressive activity in vitro. A further interesting observation may derive from the evidence that mesenchymal stem cells (MSC) of bone marrow origin suppress naïve and memory T cell responses to their cognate antigens. The observed suppression is directed at the level of T cell proliferation rather than effector function. The suppressive nature of MSC together with their role in supporting haemopoiesis makes them an ideal candidate in SCT. Potentially, MSC co-infused with the haemopoietic stem cell graft could inhibit the generation of alloreactive donor lymphocytes while facilitating engraftment.

References
geneic transplantsations was described. Several attempts have been made to improve the outcome of allogeneic hematopoietic stem cell transplantation, in order to cure a wide number of patients with no alternative therapies. The use of highly purified stem cells has significantly improved the myeloid cells engraftment however, good control of GVHD and rapid and consistent immune reconstitution, remain to be achieved. Therefore, ex vivo and in vivo strategies aimed at inducing long-term tolerance preserving Ag-specific immune responses, are desired.

We are investigating different approaches based on the immunomodulatory effect of IL-10, aimed to the adoptive transfer of regulatory T-cells in patients who received a T-cell depleted allogeneic transplantations with different HLA disparities.

The in vivo administration of human recombinant IL-10 gave conflicting results, despite the anti-inflammatory capacity and the potent suppressive effect on T-cell mediated allogeneic responses of this cytokine. This was mainly due to the fact that IL-10 can affect different types of cells with both dose-dependent and activation-state dependent effects. Conversely, we demonstrated that in vitro, the addition of IL-10 in primary MLR consistently induces alloAg specific unresponsiveness in total PBMC from mismatched or haploidentical donors. IL-10 anergized T cells preserve the ability to respond to nominal Ags, such as Tetanus toxoid and Candida albicans, and to viral Ags, such as EBV, indicating the Ag-specificity of IL-10 induced anergy. The unresponsiveness observed in proliferation is associated with a decreased frequency in alloAg specific CTLp and in a decreased ability of the anergized cells, compared to untreated cells, to induce in vitro host-specific skin tissue damage. Anergized T cell cultures contain Ag-specific Tr1 cells, which are present as precursor cells. A clinical protocol has been developed for adoptive transfer of ex-vivo IL-10 anergized T cells of donor origin to patients undergoing allogeneic hematopoietic stem cell transplantation. A pilot clinical trial has been defined and it is currently ongoing. Preliminary testing of the T cells from the haploidentical donors towards their recipients includes anergy tests, CTLp, immune responses of anergized cells and skin explant assays. Patients transplanted with haploidentical hematopoietic stem cells are enrolled to receive the infusion of anergized cells, according to a dose-escalating protocol, shortly after the stem cell engraftment. The number of patients treated is still limited and follow-up data on immune reconstitution and incidence of GVHD are preliminary. One of the limitations of this protocol has been the difficulty in reaching an effective immune reconstitution with the low number of cells, which can be infused across the high HLA disparity. This ex-vivo experimental protocol has been recently improved by the use of recipient's immature dendritic cells (iDC) treated with IL-10 and or anti-CD45 mAb, which can increase the suppressive effect of the anergized cultures by increasing the frequency of induced regulatory T cells. The use of pre-treated iDC could lead to the advantage of infusing higher number of cells, and therefore to a more rapid immune reconstitution.

As a novel approach to obtain alloAg specific tolerance in vivo, we recently investigated the possibility to induce Tr1 cells in vivo by using IL-10 together with novel immunosuppressive drugs, which do not interfere with TCR-mediated Ag recognition, such as rapamycin. This experimental model consists in the combined use of IL-10 together with rapamycin to prevent organ rejection. In this model, rapamycin and IL-10 treatment induce antigen-specific Tr1 cells which maintain self-tolerance.

Overall, these different approaches demonstrate the feasibility to modulate antigen-specific immune responses towards the induction of long-term tolerance via differentiation of Tr cells.

This work was partially founded by the Italian Ministry of Health and by Fondazione Telethon.

References
MODULATION OF APOPTOSIS

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Apoptosis is the active and programmed mechanism of cell death that plays an important role in the regulation of many biological processes. The knowledge of the molecular events governing apoptosis can help us to understand the pathogenesis of many diseases and, in addition, has the potential to open new therapeutic opportunities. Apoptosis is activated by the formation of a death-inducing signaling complex (DISC) that results in the recruitment of inducer caspases, which in turn may activate endonucleases and other proteases and in the activation of effector caspases, which results in the recruitment of inducer caspases. Apoptosis is activated by multiple and distinct pathways. It can be stimulated by an external promoter such as in the CD95/Fas receptor-mediated apoptosis upon binding of the Fas ligand. Caspase-8 is the dominant death caspase activated in this pathway and may be recruited also by the TRAIL and TNF-R1 receptors. Alternatively, a DISC designated as ‘apoptosome’ may be formed also by release into the cytoplasm of mitochondrial factors like cytochrome C and by the subsequent formation of a complex with others cytoplasmic proteins like APAF-1, CARD, ARC (apoptosis repressor with a cARD) and CIPER. The activation of this mitochondrial pathway is inhibited by Bcl-2 and other anti-apoptosis members of the Bcl-2 gene family, whereas, on the opposite, other members of the Bcl-2 gene family as Bax have been demonstrated to be direct activators. Bid activation by caspase-8 through cleavage, connects the death-receptor and the mitochondrial apoptosis pathways. Indeed activated Bid binds to Bax, leading to Bax activation and cytochrome C release from the mitochondria. More recently, a novel mechanism of apoptosis regulation involving the caspase-12 and the endoplasmic reticulum (ER) has been discovered. Caspase-12 is activated by ER stress apoptotic signals which include disruption of ER calcium homeostasis and accumulation of excess proteins in the ER. The activation of this apoptotic pathway appears to be independent from death receptor signaling and mitochondrial signals, but it’s less well known. Apoptosis is tightly connected with other vital cellular processes like cell cycle. The p53 gene protein plays a central role in this connection. p53 is a transcriptional activator of target genes like Bax and p21/WAF-1 and transcriptional repressor of other genes involved in apoptosis, but it may also activate transcription-independent mechanism of apoptosis. p53 regulates cell cycle arrest and initiation of DNA repair upon DNA damage by radiations or chemicals, eventually allowing the cell to recover from the damage and to survive. If this program fails, the simultaneously activated apoptosis pathway may reach its completion and the cell dies. Accumulation of p53 is due to stabilization of the p53 protein, which is under the control of the Mdm-2 protein. Binding to the Mdm-2 protein determines p53 degradation by the proteasome, but phosphorylation of p53 mediated via the ATM and the DNA-PK kinases, disrupts this binding and increases the p53 half-life. It is interesting to know that many signals that regulate cell proliferation are also strictly connected with apoptosis. An example of this connection is represented by the fact that the INK4a gene locus transcribes two different messengers, one coding for INK4a, the well known inhibitor of the cyclin D dependent kinase 4, whereas the second product is represented by p14ARF (Alternative Reading Frame), whose product stimulates apoptosis through the p53 pathway. Deregulation of this subtle mechanism of coordination between cell cycle control and apoptosis may lead to genetic instability, a phenomenon that may lead to progressive accumulation of genetic defects and to cancer progression. In fact, apoptosis defects are recognized as an important complement to protooncogene activation, as many deregulated oncoproteins that drive cell division also trigger apoptosis.

Many of the molecules that are directly involved in apoptosis can potentially be therapeutic targets. Cell-surface death receptors can be targeted by monoclonal antibodies, whereas therapies with antisense oligonucleotides and gene therapy approaches are generally focused on well-studied genes, such as BCL2 and p53. However, it must be underlined that, while we know that caspases serve as main effectors of apoptosis, the mechanisms involved in activation of the caspase system are less clear. Proteins like JNK, AP-1, NF-B, PKC/MAPK/ERK, and members of the sphingomyelin pathway have a profound influence on modulation of apoptosis and a better understanding of the complex interaction between different cellular programs will certainly lead to identify molecular targets for rational therapeutic strategies.

References

NEW TYROSINE KINASE INHIBITORS IN CHRONIC MYELOID LEUKEMIA

Specific tyrosine kinase inhibitors (TKIs) are rapidly developing clinical tools applied for the inhibition of malignant cell growth and metastasis formation. As a general tumor model, the chimerical Bcr-Abl protein expressed by chronic myeloid leukemia (CML) cells has constitutive tyrosine kinase activity. Imatinib, an ATP-competitive selective inhibitor of Bcr-Abl, has unprecedented efficacy for the treatment of CML. The most common imatinib resistance mechanism involves Bcr-Abl kinase domain mutations that impart varying degrees of drug insensitivity. To overcome resistance, several approaches have been studied in vitro and in vivo. They include dose escalation of imatinib, combination of imatinib with chemotherapeutic drugs, alternative Bcr-Abl inhibitors (TKIs), inhibitors of kinases downstream of Bcr-Abl, farnesyl and geranylgeranyl transferase inhibitors, histone deacetylase, proteasome and cyclin-dependent kinase inhibitors, arsenic trioxide inhibitors, histone deacetylase, proteasome and cyclin-dependent kinase inhibitors, arsenic trioxide inhibitors.

Further investigations into the molecular mechanisms of disease and how to specifically target the abnormal processes will guide the design of new treatment modalities in future clinical trials. This review highlights the development of TKIs as a specific molecularly targeted therapy and the principal mechanisms of TKIs resistance. Aspects of disease monitoring are covered as well as resistance to imatinib and strategies to overcome resistance, such as alternative signal transduction inhibitors and drug combinations. Perspectives for further development are also discussed.

This study was supported by Cofin 2003 (M. Baccarani), AIL, AIRC, Fondazione Del Monte di Bologna e Ravenna, FIRB and Ateneo 60% grants.

Acknowledgments: this work has been supported by grants AIL (Associazione Italiana contro le Leucemie) and AIRC (Associazione Italiana per la Ricerca sul Cancro).

TRANTSCRIPTIONAL THERAPY

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Local remodeling of chromatin and dynamic changes in nucleosomal packaging of DNA are key steps in the regulation of gene expression that affect proper cell function, differentiation and proliferation. Chromatin regulators by altering gene expression through histone modification might represent novel clinical anti-cancer drugs. Histone deacetylation is a distinct feature of the repressed stage of gene transcription, whereas acetylation of histones indicates the transcriptionally active genes. Transcriptional repression of retinoic acid (RA) target genes due to an aberrant recruitment of histone deacetylases (HDACs) and DNA Methyltransferases by the acute promyelocytic leukemia (APL)-associated RARα-fusion proteins is the molecular event underlying the block at the promyelocytic stage of myeloid differentiation and leukemogenesis in this acute myeloid leukemia (AML)-M3 FAB subtype.1-3 Paradoxically, APL is also the most striking clinical success of a RA-based differentiation therapy in human neoplasia, and has become the molecular paradigm for therapeutic approaches utilizing differentiating agents.1-3 Indeed, pharmacological doses of RA can release the HDAC repressor complex and recruit the multisubunit histone acetyltransferases (HAT) activation complex on RA target genes, resulting in terminal differentiation of PML/RARα-positive APL blasts, which account for more than 90% of APLs.1-3 While non-APL AML subtypes are not sensitive to retinoids,1 an altered expression and activity of enzymes with chromatin remodeling function such as the HDACs, HATs or of their binding partners is present in other AML subtypes. Indeed, as a result of the t(8;21) chromosomal translocation associated with AML-M2, the HAT-interacting domain of the hematopoietic transcription factor AML1 is lost and replaced by region of ETO interacting with a protein complex containing HDAC activity. The resulting AML1-ETO fusion protein also forms oligomeric structures that lead to transcriptional repression of AML1 an RA signaling pathways and block of myeloid differentiation.2,4 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 subtypes.5,6 A structure function analysis of the MLL-CBP product of the t(11;16) translocation demonstrated that fusion of both the bromodomain and HAT domain of CBP to the amino portion of MLL induces full trans-
formation and the leukemic phenotype in vivo. In addition, recent experimental evidence indicate that the RA signaling pathway is involved in normal and pathological myelopoiesis. Functional RA responsive elements are present in the promoter region of transcription factors involved in granulocytic myelopoiesis, suggesting that a regulated expression of RA responsive genes may be crucial for effective myeloid differentiation. In agreement, with this hypothesis are our observations indicating that in AMLs, regardless of their underlying genetic alteration, the RA-signaling pathway is constitutively repressed through an HDAC-dependent mechanism.

Therefore, it appears that in AMLs different genetic alterations resulting in common patterns of deregulated gene expression may lead to differentiation block and myeloid leukemogenesis. Drugs modulating acetylation status of histones might represent a novel therapeutical approach to reprogram leukemic cells to differentiation and to eradicate the neoplastic cells. Interestingly, the global chromatin remodeling activity of these inhibitors affects only few (4-10%) selected genes. This specificity, along with the low in vivo toxicity of these compounds, raises the possibility of their clinical use. Clinical trials are currently evaluating their potential use for a transcriptional therapy of neoplasia and leukemia.

References

BEST ABSTRACTS

BEST-01
FISH DIAGNOSIS OF CIZ TRANSLOCATIONS IN ACUTE LYMPHOPROLIFERATIVE LEUKEMIA


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Rearrangements of 12p13/CIZ with the TET (TLS, EWSR1, and TAF15) proteins, namely TAF15/17q11 or EWSR1/22q12 identifies a clinical-hematological entity among acute lymphoblastic leukemia (ALL), in both children and adults. We collected seven cases of ALL showing a t(12;17)(p13;q11) (five cases) or a t(12;22)(p13;q12)(two cases). Patients with t(12;17) were males and females, aged between 4–44. Immunophenotype showed positivity for CD34 and CD19 in all cases. HLA-DR and CD22 were positive in the four cases tested. In 4/5 patients at least one myeloid antigen (CD13 and/or CD33) was expressed. Only the pediatric case showed expression of CD10 in 30% of marrow blasts. The two patients with t(12;22) were one male and one female, 4 and 29 years old, respectively. In both cases there was a coexpression of myeloid (both CD13 and CD33) antigens, and the pediatric patient was CD10 positive. All patients achieved complete remission during intensive chemotherapy. Six patients are alive with a median follow-up of 48.5 months (range 8–89). Appropriate diagnostic tools for metaphase and interphase FISH were developed. BAC433J6 spanning the 12p13/CIZ breakpoint in all cases is useful for interphase-FISH investigations at diagnosis or during disease monitoring. DNA probes generated by LD-PCR for the 5' (probe 8.2) and the 3' (probe 9.6) of CIZ are helpful to show translocation of the 5'CIZ to both der(17) and der(22), with the 3'CIZ retained on der(12). These probes were adapted to metaphase-FISH in order to confirm 12p13/CIZ involvement. A paradigmatic example is represented from one case of ALL with a t(10;12)(p12;p13) at karyotype, in which a complex genomic change involved the CIZ gene with insertion of the 5'end within band p13 of chromosome 19. FISH is a unique tool to diagnose chromosomal recombinations of CIZ associated acute lymphoblastic leukemia.

This work was partially supported by CNR-MIUR and FIRB.

BEST-02
TRAIL DECOY RECEPTORS,TRAIL-R3 AND TRAIL-R4, MEDIATE RESISTANCE OF ACUTE MYELOID LEUKEMIA CELLS TO TRAIL


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The TNF related apoptosis-inducing ligand (TRAIL), also known as Apo2 ligand (Apo2L), is a member of the structurally related TNF family of cytokines. TRAIL can bind to at least four different cell surface receptors: TRAIL-R1 (also called DR4) and TRAIL-R2 (also called killer /DR5) are receptors with two cysteine-rich extracellular ligand-binding domains and a cytoplasmic death domain that signals upstream caspase activation, TRAIL-R3 (also called DcR1 or TRID) and TRAIL-R4 (also called DcR2 or TRUNDD) are GPI-anchored surface receptors lacking a functional active intracellular death domain. These two receptors are thought to protect cells from TRAIL-mediated apoptosis as decoy receptors by competing with the functionally active TRAIL R1 and TRAIL R2 for binding to TRAIL. The unique features of TRAIL, with respect to CD95 L and TNF-α, is considered its ability to induce apoptosis in a variety of malignant cells, including several of hematopoietic origin, displaying no toxicity on normal cells and tissues. The antitumor activity of TRAIL was explored in hematologic malignancies and few studies have evaluated TRAIL susceptibility of AML cells, showing a low sensitivity of AML blasts to the cytotoxic effects of TRAIL; however, it is not clear whether the mechanism of resistance to TRAIL is constitutive or inductive. The aim of our study was to investigate the biologic activity of TRAIL on AMLs and to determine the mechanism of their resistance to apoptosis. In our hands, the blasts cells isolated from the large majority of patients with AML are resistant to apoptosis induction of TRAIL (annexin V test), showing a peculiar expression pattern of TRAIL receptor (TRAILRs)
expression. TRAILR expression was explored at protein level using specific monoclonal antibodies. We found that TRAIL R3 and TRAIL R4 are expressed in the majority of cases, TRAIL R1 on about 30% of cases, while TRAIL R2 only in two cases out 44 analyzed. In spite the heterogeneity of the cases analyzed, pertaining to all FAB subtypes, it is possible to correlate TRAILR expression pattern with different AML subgroups. Interestingly the expression of TRAIL-R1 is limited to AMLs with monocytic features, as supported by the presence of CD14 and CD11b antigens. AML M3 express TRAIL-R3 and TRAIL-R4 in the majority of cases, while TRAIL-R1 and TRAIL-R2 were usually undetectable. Membrane TRAIL-bound was observed in the majority of APL patients (4/5), while it was observed only in a low percentage of the other AML subtypes. Given the results we explored the effect of fusion protein PML/RAR-α on TRAIL/TRAILRs expression and on the modulation of TRAIL sensitivity. Using U937 as a cellular system we showed that induction of the PML/RAR-α protein in these cells was associated with a downmodulation of both TRAIL-R1 expression and TRAIL-mediated cytolysis (i.e., U937 cells expressing PML/RAR-α became resistant to TRAIL). Treatment of APL blast with retinoic acid was associated with a marked downmodulation of TRAIL expression and with no modification of the sensitivity to TRAIL (i.e., the cells remained resistant to TRAIL-mediated cytolysis). According to these results we suggest that AMLs are resistant to TRAIL due to the expression of TRAIL decoy receptors and to the down modulation of TRAIL-R1. Furthermore, the expression of TRAIL membrane bound on the surface of APL blasts may confer a condition of immunologic privilege to these cells.

**BEST-03**

**BONE MARROW STROMAL CELLS (BMSC) FROM ACUTE MYELOID LEUKEMIA PATIENTS: INTERACTION WITH NATURAL KILLER CELLS**

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Normal blood cell differentiation needs the interaction between hematopoietic precursors and bone marrow stromal cells (BMSC). It is thought that abnormalities occurring during such interactions may contribute to the oncogenesis in acute leukemias, making of interest the definition of the functional role of BMSC in normal hemopoiesis and neoplastic transformation. In this study, BMSC were obtained from 10 patients suffering from acute myeloid leukemia (AML), six M0/M1 two M2, and two M5 (according to the FAB classification), 8 out of 10 in post-chemotherapy complete remission. BMSC were obtained from all of these patients and maintained in culture for two months. These cells were analysed for the expression of a panel of surface markers at different culture passages (from 1 to 4). BMSC were CD44+, CD73a+ CD73b+ CD105+, β1 integrin+, ICAM1+, HLA-I+, HLA-I+ (variable proportions), CD34– CD14– CD45– CD31–, and they could express the stress-inducible MHC-related molecules MIC-A and the UL16 (induced at the surface of cells infected by cytomegalovirus) binding protein ULBP3. These molecules are reported ligands for the NKG2D receptor expressed by Natural Killer (NK) and CD8+ T lymphocytes, effector cells that are thought to play a role in host defence against tumors. NK cells have also been shown to regulate normal differentiation of hemopoietic precursor into the myeloid or lymphoid cell lineage. Moreover, it has been stated that NK cells are not able to damage autologous cells, as they receive negative signals through inhibitory receptors, including killer Ig-like receptors (KIR) or C-type lectin inhibitory receptors (CLIR), which bind to HLA-I discrete alleles. Surprisingly, we found that autologous IL2-activated, but not freshly isolated, NK cells lysed BMSC, while T lymphocytes did not kill self or non-self BMSC. Binding of ICAM-1 expressed by BMSC to its receptor, the integrin LFA-1, expressed by NK cells, plays a key role in BMSC/NK interaction. More importantly, NKG2D/MICA and/or NKG2D/ULBP3 engagement is responsible for the delivery of lethal hit. Conversely, it appears that HLA-I molecules do not protect BMSC from NK cell-mediated injury. Taken together, these data suggest that NK cells, when activated as it may occur during the first response to viral infections, are able to eliminate BMSC, thus altering the normal interactions with hemopoietic precursors and possibly affecting their differentiation. This mechanism might also contribute to the development of aberrant precursors as observed in acute leukaemias.
Sequences were then matched in databases to identify means of an unbiased inverse-PCR strategy. Domain sequences of monoclonal Ig were obtained when the heavy chain sequence belonged to the rearranged germline V, D and J segments. The variable region was most closely related (94% identity) to the VH3-30 germline gene (VH3 family) to D2-2 and JH4b segments, whereas the V region was derived (97% identity) from the germline VJL6 (VIII) rearranged to J15. Partial protein sequence of serum cryoprecipitate confirmed the correct identification of the monoclonal sequences. A previously reported model of murine cryocrystalglobulinemia suggested that acquisition of positively charged amino acids within the VH domain (positions 6 and 23) could influence cryoprecipitation. Analysis of human monoclonal IgG2 BEL heavy chain variable domain did not confirm these observations. Protein modelling and site-directed mutagenesis studies will be performed to clarify the role of amino acid substitutions in immunoglobulin crystal-like aggregation.

Cryocrystalglobulinemia is characterized by a monoclonal immunoglobulin that is both cryoprecipitable and crystal-forming (cryocrystalglobulin). It occurs in humans as a rare complication of lymphoproliferative disorders. The monoclonal immunoglobulins may crystallize in serum and synovial fluid and are responsible for both microcrystalline synovial inflammation and occlusive vasculopathy in kidneys and skin. At present, there are no reports on sequence analysis and tertiary structure of human cryocrystalglobulins. In this study, we established the first light and heavy chain variable region sequences of a human cryocrystalglobulin (BEL) from a patient with severe microcrystalline arthropathy, cutaneous purpura and sacroiliitis. Serum cryoprecipitate was composed of unbound monoclonal IgG and was derived from the VH3-30 germline gene (VH3 family) and revealed homogeneous crystals under light microscopy. Extra and intracellularly crystals were also evident in synovial fluid. Analysis of bone marrow aspirate showed 4% monoclonal plasma cell infiltration. Total RNA was extracted from Ficoll-separated bone marrow mononuclear cells and complete heavy (VH) and light chain (VL) variable domain sequences of monoclonal IgG were obtained by means of an unbiased inverse-PCR strategy. Sequences were then matched in databases to identify rearranged germline V, D and J segments. The heavy chain sequence belonged to the ≥2 class and its variable region was most closely related (94% identity) to the VH3-30 germline gene (VH3 family) rearranged to D2-2 and JH4b segments, whereas the V region was derived (97% identity) from the germline VJL6 (VIII) rearranged to J15. Partial protein sequence of serum cryoprecipitate confirmed the correct identification of the monoclonal sequences. A previously reported model of murine cryocrystalglobulinemia suggested that acquisition of positively charged amino acids within the VH domain (positions 6 and 23) could influence cryoprecipitation. Analysis of human monoclonal IgG2 BEL heavy chain variable domain did not confirm these observations. Protein modelling and site-directed mutagenesis studies will be performed to clarify the role of amino acid substitutions in immunoglobulin crystal-like aggregation.

The mechanism of action of rituximab in vivo has been studied using a new B lymphoma model homing in the haematopoietic tissues and lymph nodes of immunocompetent mice best mimicking human B-NHL. The human CD20 cDNA was introduced into the 38C13 murine B lymphoma cell line by retroviral infection. The transduced and selected CD20+ cells stably expressed the CD20 protein and produced tumours in vivo with the same kinetics as wild type cells. Inoculation of 4×10^3 38C13-CD20+ intravenously into syngeneic C3H/HeN mice led to the development of tumour masses in the spleen, bone marrow and lymph nodes, detectable from 15 days after PCR, and with a median survival times of 22–23 days. Treatment with 250 µg rituximab i. p. given one day, or up to 10 days after tumour, cured 100% of animals, with disappearance of tumour documented by immunohistochemistry and PCR analysis. Rituximab did not inhibit 38C13-CD20+ cell growth in vitro. Depletion of both NK cells and neutrophils did not affect the therapeutic activity of rituximab in vivo. Similarly removal of phagocytic macrophages using cobra venom factor. These data demonstrate that complement is required for the therapeutic activity of rituximab in vivo. The protective activity of the antibody was completely abolished after complement depletion with cobra venom factor. These data demonstrate that complement is required for the therapeutic activity of rituximab in vivo in an immunocompetent murine model of lymphoma homing in lymph nodes.
NK CELL CONDITIONING TO T CELL-REPLETE MISMATCHED BONE MARROW TRANSPLANTATION PROTECTS THYMIC STRUCTURE AND FUNCTION

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Conditioning regimens which include alloreactive NK cells are a new strategy for improving immune reconstitution after mismatched bone marrow transplantation (BMT) as they allow for the concomitant transfer of high doses of donor T cells without causing GVHD (Science 2002;295:2097). Even in the overt absence of acute systemic GVHD, the NK conditioning itself, or the transfer of allogeneic T cells might still cause acute thymic GVHD, i.e., damaged stroma, and impaired thymopoiesis and selection of the T cell repertoire. To test this assertion, recipient mice (H-2b) were lethally irradiated and transplanted with mismatched bone marrow cells (H-2d) in the presence or absence of a lethal dose of allogeneic T lymphocytes (H-2d; 1.5×10⁷). Some of the recipient mice were conditioned with irradiation and donor alloreactive NK cells (Ly49A/G2+, Ly49C/I−). Infusion of NK cells prevented GVHD in all recipients of T-replete BMT, which otherwise died of GVHD. Conditioning with NK cells followed by T-replete bone marrow transplantation did not affect total thymocyte numbers or result in the morphological changes that are typical of thymic GVHD, i.e., loss of the corticomedullary demarcation, alterations in the composition and orientation of epithelial stroma, and general thymic involution. Analysis of intrathymic T lineage development demonstrated normal maturation progression from the most immature thymocytes to phenotypically mature intrathymic T cells. Examination of the relative distribution of all thymocyte subpopulations did not reveal any of the changes commonly observed with acute thymic GVHD, i.e., a loss of double positive thymocytes and an increase in both donor bone marrow-derived mature T cells and immature precursor cells (Blood 2000;96:347). These results demonstrate that the use of an alloreactive NK-based conditioning regimen in conjunction with the adoptive transfer of large numbers of allogeneic T cells affects neither the architectural organization nor the cellular composition of the thymic stromal compartment nor does it impair regular thymopoiesis. Conditioning with alloreactive NK cells may, therefore, provide a safe strategy to allow for adoptive immunotherapy without endangering thymic T cell reconstitution after mismatched BMT.
CO-01
GENE EXPRESSION PROFILING OF NORMAL AND MALIGNANT CD34- DERIVED MEGAKARYOCYTIC CELLS
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CD34+ hematopoietic progenitor cells taken from the bone marrow of Essential Thrombocythemia (ET) patients and healthy subjects were induced to differentiate along the megakaryocytic lineage in liquid suspension cultures by continuous addition of 100ng/mL of thrombopoietin. Gene expression profiles of bone marrow CD34-derived megakaryocytic cells (MKs) were compared in patients with ET and healthy subjects utilizing oligonucleotide microarray analysis to identify differentially expressed genes and disease-specific transcripts. We found that pro-apoptotic genes such as BAX, BNIP3 and BNIP3L were downregulated in ET MKs together with genes that are component of the mitochondrial permeability transition pore complex (a system with a pivotal role in apoptosis). Conversely, anti-apoptotic genes like IGF1-R and CFLAR were upregulated in the malignant cells, as was the SDF1 gene, which favors cell-survival. Based on the array results, we characterized apoptosis of normal and ET MKs by time-course evaluation of Annexin V and sub-G1 peak DNA stainings of immature (day 8) and mature (day 14) MKs after culture in serum-free medium with an optimal thrombopoietin concentration (100 ng/ml) and (Annexin V-positive MKs only) with decreasing thrombopoietin concentrations (100, 10, 1, 0.1 ng/ml). We found that ET MKs were more resistant to apoptosis than their normal counterparts. We conclude that imbalance between proliferation and apoptosis seems to be a relevant step in malignant ET megakaryocytepoiesis.

CO-02
MOLECULAR CHARACTERIZATION OF HUMAN HEMATOPOIETIC CD34- AND CD34+ STEM CELLS
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The existence of murine and human CD34- lineage- (Lin-CD34-) hematopoietic stem cells (HSC) capable to engraft myeloablated hosts has recently been demonstrated. CD34- HSC are kinetically quiescent, can be induced to express CD34 and to proliferate, in vitro, upon cytokine treatment and their hematopoietic activity depends on the expression of CD34. Furthermore, xenotransplant studies have shown that the expression of CD34 on human HSC is reversible. In this paper, we attempted to clarify the molecular mechanisms governing the different biological behaviour of different subsets of HSC, according to the expression of CD34 molecule. To this end, we used the DNA microarray technology to evaluate the expression profile of Lin-CD34-, Lin-CD34+ and Lin+CD34+ HSC. The analysis of gene categories differentially expressed showed that the acquisition of CD34 is associated with cell cycle entry and general metabolic activation, such as DNA, RNA and protein synthesis. Moreover, the significant up-regulation in CD34- cells of pathways inhibiting HSC proliferation induces a strong differential expression of cyclins, CDK inhibitors and growth-arrest genes. The analysis of transcriptional factors shows that the expression of CD34 results in the up-regulation of self-renewal- and lineage-commitment-related genes. The preferential expression in CD34+ cells of genes supporting the mobilization of HSC and their homing to the bone marrow, such as chemokine receptors and integrins, gives the molecular basis for the higher engraftment capacity of CD34+ cells. Conversely, CD34- cells express preferentially genes related to neural, epithelial, and muscle differentiation. Thus, the molecular expression profiles reported here contribute to a more detailed understanding of HSC biology and function.
**CO-03**

**GENE EXPRESSION PROFILING OF HUMAN HEMATOPOIETIC STEM CELLS AND TERMINALLY DIFFERENTIATED MYELOID CELLS**

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The comparison of the gene expression profiles of hematopoietic stem cells (HSC) and terminally differentiated myeloid cells can be remarkable for the molecular reconstruction of myeloid differentiation programs and for the identification of new lineage specific markers. In this work we studied the gene expression profile of CD34+ HSC and normal terminally differentiated myeloid cells (monocytes, neutrophil and eosinophil) using Affymetrix HG-U95Av2 GeneChip array technology. The global gene expression analysis showed a significantly higher complexity of mRNAs in CD34+ HSC population compared with terminally differentiated myeloid cells. The functional analysis of the differentially expressed genes, performed using Gene Ontology (GO) Mining Tool software, revealed a general metabolic activation in CD34+ cells (activation of DNA replication, transcription and RNA processing, ribosome and protein synthesis), while the majority of the preferentially expressed genes in mature leucocytes were found belonging to the defense immunity GO category. According to these preliminary observations, we found a preferential expression of G1/S cyclins and CDKs in CD34+ cells, whereas CDK Inhibitors and protein synthesis, while the majority of the preferentially expressed genes in mature leucocytes were found belonging to the defense immunity GO category. According to these preliminary observations, we found a preferential expression of G1/S cyclins and CDKs in CD34+ cells, whereas CDK Inhibitors and genes involved in immune response, such as inflammatory cytokines and chemokines receptors, cytotoxic granules proteins, oxidative burst enzymes, MHC class II molecules and components of INF gamma pathway are up-regulated in differentiated myeloid cells. Moreover, we found up-regulated in CD34+ cells the expression of self-renewal and lineage commitment-related transcription factors (TFs), whereas leucocyte samples showed a preferential expression of TFs involved in maintenance of the terminally differentiated phenotypes. This work provides a strong molecular support to essential properties of the HSC and of terminally differentiated myeloid cells; moreover, in vitro functional assays will allow us to identify the correlations between changes in gene expression occurring in the commitment phase and the activation of the myeloid differentiation program.

**CO-04**

**EXPRESSION OF TRANSCRIPTIONAL COREPRESSOR NCOR OR ITS RECEPTOR INTERACTION DOMAIN AFFECTS LIGAND BINDING TO WILD TYPE RETINOIC ACID RECEPTOR α AND PML/RARα**

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Acute promyelocytic leukemia (APL) is associated with reciprocal chromosomal translocations involving the RARα locus with either PML or more rarely PLZF. Such fusion proteins inhibit physiological retinoid signalling via the RAR/RXR pathway and this action is linked to their oncogenic activity which is achieved through aberrant recruitment of nuclear corepressor molecules such as NCoR or SMRT and histone deacetylases. A unique feature of PMLRARα expressing APL is its sensitivity to retinoic acid (RA) therapy, which induces remission by promoting cellular differentiation. To investigate the molecular mechanisms of leukemogenesis by PMLRARα and of acquired RA resistance we addressed the biological role of the interaction of transcriptional regulators with nuclear receptors (NR). To this end we expressed the transcriptional co-repressor NCoR or its interaction domains (IDC and IDN) into COS-1 cells (in co-transfection with RARα and PML/RARα), U937 (expressing RARα) and NB-4 (expressing PMLRARα). In these cells we analyzed: i) the molecular interactions of the above mentioned molecules with radioactive RA ([H3]-RA) through HPLC; ii) the effects on RA target promoters and iii) differentiation status. An IDC with three aminoacids mutated to alanine in the receptor interaction domain (IDC-mut10) or an antisense IDN (IDNAS) were also used as controls. The results obtained showed that the over-expression of NCoR or of IDC, its domain that interacts with nuclear receptors, strongly increases the RA-binding to RARα and PML/RARα. Moreover, IDC increased PMLRARα binding to retinoic acid also when stably transfected into U937 induced to express PMLRARα (U937-PR9). In contrast, the over-expression of IDN (another NCoR domain that interacts with nuclear receptors), IDC-mut10, IDNAS and of transcriptional co-activators TIF2 and NSD1 did not significantly modify the capacity of RARα and PML/RARα to bind RA. NCoR, IDC and IDN, modified the conformation of the RA receptors, as shown by tryptic digestion patterns of RARα and PML/RARα. In vitro binding assays with GST
and his-tagged fusion proteins confirmed these in vivo results. In fact, IDC-his peptide caused a dose dependent increase in GSTRAR binding to [3H]-RA. The same result was obtained when a SMRT receptor interaction domain (GSTIDII) was used, while the SMRT PLZF interaction domain (GSTPID2) or an empty histidine vector did not modify RA binding pointed out the specificity of this effect. IDC, when transiently transfected into the NB4 or U937 cell lines, increases the RA induced transcriptional activity of a Luc-reporter gene controlled by a β-RARE. Instead the transient transfection of NCoR or IDN slightly change this transcriptional activity. Finally, NB4-R4 cells (an NB-4 clone resistant to RA) transfected with IDC overcome the differentiation block and are able to differentiate following RA treatment. Our data highlight a novel role for transcriptional corepressors or peptides representing their interaction domains in modulating ligand binding to nuclear receptors. These results help to analyse the molecular events involved in RA resistance and in aberrant nuclear receptor interactions, in order to elucidate new therapeutic strategies for handling leukemias characterized by aberrant NRs.

**CO-05**

**THE COMBINATION WITH MEK1 INHIBITOR ENHANCES ARSENIC TRIoxide INDUCED APOPTOSIS OF ACUTE LEUKEMIA BLASTS**

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According to recent laboratory studies, the blast cells of most acute myelogenous leukemias (AML) including acute promyelocytic leukemia (APL) show constitutive activation of extracellular signal-regulated kinases 1/2 (ERKs 1/2) as well as of the kinases immediately upstream of ERK, known as mitogen-activated protein (MAP)/ERK kinases (MEKs). Furthermore, we and others have demonstrated that down-modulation of MEK1 phosphorylation inhibits the proliferation and induces apoptosis of primary AML blast cells. In this study, we firstly aimed at investigating whether the combination of arsenic trioxide (ATO) with agents that block the phosphorylation of MEK1 can potentiate the anti-leukemic action of ATO in APL. We then investigated whether this combination is capable to enhance apoptosis of non APL acute leukemia primary blasts. For our purposes we studied parental NB4 cell line, an arsenic-resistant NB4 subline (NB4-AsR) derived in our laboratory from the NB4 cell line, primary blast cells of typical hypergranular APL (M3) carrying PML/RARα fusion transcript, primary blast cells of AML (M1 or M2) carrying 47, XX, +8 or 46, XX inv (16), of acute monocytic leukemia (M5), of acute lymphocytic leukemia carrying 46, XX, del (11)(q23). Leukemic cells were pre-treated with PD98059 (Cell Signaling Technology, Beverly, MA, USA) 10, 20 or 40 μM or PD184352 (kindly provided to us by Dr J. S. Sebolt-Leopold, Cancer Molecular Sciences, Pfizer Global Research & Development, Ann Arbor, MI, USA) 1 or 2 μM, and then treated with ATO 0.5–2 μM. We found that leukemia cells exploit the Ras–MAPK activation pathway to phosphorylate at Ser112 and to inactivate the pro-apoptotic protein Bad, delaying ATO-induced apoptosis. Both in APL cell line NB4 and in primary blasts, the inhibition of ERK1/2 activity and of Bad phosphorylation by MEK1 inhibitors enhanced and accelerated apoptosis in ATO-treated cells. NB4-AsR showed stronger ERK1/2 activity (2.7 fold increase) and Bad phosphorylation (2.4 fold increase) compared to parental NB4 cells in response to ATO treatment. Upon ATO exposure, both NB4 and NB4-AsR cell lines doubled protein levels of the death antagonist Bcl-XL but the amount of free Bcl-XL that did not heterodimerize with Bad was 1.8 fold greater in NB4-AsR than in the parental line. MEK1 inhibitors dephosphorylated Bad and inhibited the ATO-induced increase of Bcl-XL, overcoming ATO resistance in NB4-AsR. Synergism, additive effects, and antagonism were assessed using the Chou-Talay method and CalcuSyn software (Biosoft, Ferguson, MO). PD + ATO combination synergized for the induction of apoptosis primarily in arsenic resistant but also in parental NB4 cells. Furthermore, the combination PD + ATO significantly increased the ATO-induced apoptosis in primary acute leukemia blasts (p<0.001). These results may provide a rationale to develop combined MEK1 inhibitors plus ATO therapy in APL and in other types of acute leukemia.

**CO-06**

**ROLE OF NF-KB IN THE PREVENTION OF ETOPOSIDE-INDUCED APOPTOSIS IN K562 CELL LINE**

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Resistance to apoptosis plays an essential role both in tumorigenesis and in chemotherapy failure. The inability of cytotoxic drugs to induce effective apop-
tosis in cancer cells is based on their degradation/inactivation and/or abnormal signal transduction pathways that activate anti-apoptotic programs. The constitutive active PI3K/Akt and NF-κB pathways mediates strong anti-apoptotic signals in many cancer cells. NF-κB is a dimeric protein composed of members of the Rel/NF-κB family, including RelA, RelB, c-Rel, p50 and p52. NF-κB dimers are retained into the nucleus by the inhibitory protein IkB. Different stimuli determines IkB phosphorylation which is ubiquitinated and degraded by the 26S proteosome. Free NF-κB translocates into the nucleus where it controls transcription of genes involved in transformation and protection of apoptosis. CML is a myeloproliferative disorder characterized by the expression of the t(9;22) translocation which encode for the chimeric fusion protein Bcr-Abl. The development of the specific inhibitor of the kinase activity of Abl, Imatinib, has completely revolutionized the therapy and the prognosis of the chronic phase of CML. Unfortunately, Bcr-Abl inhibition does not affect the blast phase of the disease, where traditional chemotherapy appear to be the only therapeutical approach. K562 is a cell line derived from a CML blast crisis which is particularly resistant to many traditional chemotherapeutic agents such as the topoisomerase inhibitor Etoposide. In this work we have first evaluated whether the exposure to Etoposide activates NF-κB in K562. Cells have been treated with Etoposide for 2 hours and then nuclear extract have been obtained. The transcriptional activity of NF-κB has been evaluated with an ELISA method that quantified the binding of nuclear NF-κB to its specific DNA binding motif. NF-κB activity is increased of 2.1 and 3.2 times when cells are exposed to 10 and 100 microM of Etoposide.

Etoposide activates NF-κB in K562. Cells have been treated with Etoposide for 2 hours and then nuclear extract have been obtained. The transcriptional activity of NF-κB has been evaluated with an ELISA method that quantified the binding of nuclear NF-κB to its specific DNA binding motif. NF-κB binding activity is increased of 2.1 and 3.2 times when cells are exposed to 10 and 100 microM of Etoposide respectively. When cells have been pretreated with the NF-κB inhibitors Resveratrol (30 μM), MG-132 (1 μM) and Bay (5 μM), NF-κB has not been activated by Etoposide. When K562 are exposed to 100 μM Etoposide, apoptosis (evaluated with a Cell Death Detection ELISA method, which quantified the release of histone-bound DNA) is undetectable. The exposure to the NF-κB inhibitors MG-132, Bay and with a less extent to Resveratrol determines a moderate induction of apoptosis. Treatment with both NF-κB inhibitors and Etoposide causes a marked increase of apoptosis. To prove that NF-κB inhibition is the causal mechanism by which cells becomes sensitive to Etoposide induced apoptosis, we have developed a stable K562 cell line expressing a SuperRepressor-IkB. SR-IkB is a mutated IkB protein which can not be degraded. This stable IkB blocks NF-κB in the cytoplasm. In SR-IkB stable cell lines, Etoposide does not activate NF-κB and acts as an apoptosis inducer. These data suggest that the activation of NF-κB may be responsible of the resistance to Etoposide induced apoptosis in K562 cell line and that NF-κB inhibitors may restore sensitiveness to this conventional cytotoxic agent.

CO-07
EVIDENCE OF BIASED USAGE OF IMMUNOGLOBULIN VARIABLE GENES IN AIDS-RELATED NON-HODGKIN’S LYMPHOMA: IMPLICATION FOR THE PATHOGENESIS OF THE DISEASE

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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 AIDS-patients. AIDS-related NHL (AIDS-NHL) derive from mature B-cells and are phenotypically and histogenetically related to germinal center (GC) or post-GC B-cell. AIDS-NHL are classified into: (i) diffuse large B-cell lymphoma (AIDS-DLBCL); (ii) Burkitt/Burkitt-like lymphoma (AIDS-BL/BLL); (iii) plasmablastic lymphoma of the oral cavity (AIDS-PBL); and (iv) primary effusion lymphoma (AIDS-PEL). Analysis of immunoglobulin variable (IgV) genes used by B-cell malignancies reveals the cell origin of the tumor and its clonal history following neoplastic transformation. Here we investigated a panel of 67 AIDS-NHL, including 30 AIDS-DLBCL, 21 AIDS-BL/BLL, 6 AIDS-PBL and 10 AIDS-PBL for usage, mutation frequency and intratumoral heterogeneity of clonal IgV rearrangements. Moreover, to ascertain the role of antigens and/or superantigens in AIDS-NHL pathogenesis, we analyzed the mutational pattern and CDR3 structure of IgV heavy (H) and light (L) chain genes. Results where compared to a database of 200 DLBCL IgV rearrangements and to the normal B-cell repertoire. A functional IgV rearrangement was found in 58/65 (89.2%) cases, a functional IgVκ chain rearrangement was identified in 17/38 (44.7%) cases and a functional IgV lambda chain rearrangement in 21/38 cases (55.3%). Despite exhaustive analysis by multiple PCR strategies, 10% of cases showed only non functional IgVH and/or IgVL rearrangements, suggesting a cellular origin from B-cells that have lost the ability to express antigen receptors. Somatic mutations in IgVH and/or IgVL genes were found in 58/67 (88.1%) AIDS-NHL. The average mutation frequency was 9.42% (median 7.50%, range 2.04–23.3%) for IgVH genes and 5.42% for IgVL genes.
Finally, at variance with NHL of immunocompetent host, the presence of intracranial heterogeneity is a rare finding in AIDS-NHL, suggesting a derivation from B-cells that have concluded the GC-reaction.

**Background:** the Ets variant gene 6 (ETV6/TEL), a transcriptional repressor, is rearranged in the majority of haematological patients with 12q13 translocations and fused to a number of different partners. We previously identified TEL/ARG, a novel fusion transcript consisting of the oligomerization domain of TEL and the catalytic domain of the tyrosine kinase Abelson-related gene (ARG), in a case of acute myeloid leukaemia carrying a t(1;12)(q25;p13). Aim of the study was to investigate the transforming activity of TEL/ARG, focusing on interactions with the cytoskeletal elements, as described for the homologue BCR/ABL. Methods: we fused the FLAG sequence to the TEL/ARG cDNA and cloned this construct in the bicistronic lentiviral vector pRRLPPT. CMV.iresGFPpre at 5' terminal of the IRES sequence. Results: we analysed the subcellular localization of the chimeric proteins in transduced human 293T fibroblasts by immunofluorescence using an anti-FLAG antibody; we found that TEL/ARG was cytoplasmic and co-localized with the actin. Moreover, its expression in 293T, monitored by GFP expression and western-blot analyses, induced a morphologic change to fibroblasts, which underwent cell rounding and detachment from the tissue culture plastic. Conclusions: TEL/ARG transcript seems to induce abnormalities of cytoskeletal function, a potentially important mechanism for its oncogenic activity. It will be interesting to investigate the effects of TEL/ARG expression in haematopoietic precursor cells, where other authors described a role of the homologue BCR/ABL in inducing cytoskeletal abnormalities and altered adhesion to extracellular bone marrow matrix protein.
Regulatory mechanisms governing homing and engraftment of hematopoietic stem cells are poorly understood: they depend on a complex interplay between chemokines, cytokines, growth factors and adhesion molecules in the intricate architecture of bone marrow microenvironment. Extracellular nucleotides were recently reported as potent proliferative factors for hematopoietic stem cells; they are also emerging as chemotactic factors for different cell types, including vascular endothelial cells, arterial smooth muscle cells and neutrophils; they also regulate the trafficking of specific dendritic cell populations. In this study we asked if the extracellular nucleotide UTP would modulate the migration of hematopoietic stem cells in response to the chemotactic peptide CXCL12/SDF-1α. Very low concentration of UTP (at 10 μM) significantly improved stem cell migration, assayed with the dual-chamber migration system and evaluated with cell counting and clonogenic assays. Phenotypical analysis of CXCR4 antigen showed that SDF-1α gradients mainly attract hematopoietic stem cells expressing high levels of this receptor; whereas, migrating cells, previously treated with UTP, showed no substantial differences in CXCR4 level, compared to non-migrating cells. Furthermore we evaluated actin polymerization as index of stem cell capacity to respond to chemotactic stimulation and to home to the BM microenvironment. UTP-treated CD34+/CD56+ cells, once stimulated with SDF-1α, showed a significant increase in actin polymerization, as indicated by the enhanced fluorescence intensity of FITC-phalloidin. Moreover, xenogenic transplant studies showed that short-term pre-incubation with UTP significantly increased engraftment efficiency in nonobese diabetic/severe combined immunodeficiency mice. Taken together, our data suggest that UTP may affect HSC migration and homing in the bone marrow, in synergy with the chemotactic peptide SDF-1α, perhaps activating a CXCR4–independent pathway.

It has been previously demonstrated that purified CD34+ cells induce allogeneic T cell proliferation and generation of cytotoxic T cells. In this study, we addressed the hypothesis of whether CD34+ cells may activate also NK cells. To test whether CD34+ cells could directly activate NK cells, immunomagnetically purified CD3-CD56+ NK cells were cultured with either purified allogeneic CD34+ or CD14+ cells, at a ratio of 1:1 to 2:1, or with high dose (1000 U/ml) IL-2 for 3-4 days. NK activity was evaluated as their ability to induce the lysis of either NK-sensitive K562 or NK-resistant Daudi target cells in a standard Cr51 lysis assay. CD34+ cells induced significantly greater NK activation than monocytes, as measured as lysis of K562 cells (50±14% vs 15±17%, respectively) (n=4, p=0.02). We then tested whether coculture of purified NK cells with CD34+ cells would result in the lysis of NK-resistant Daudi cells (LAK activity). While LAK activity was negligible before culture, it substantially increased upon culture with CD34+ cells, but not monocytes (53±11% vs 22±16%, respectively) (n=6, p=0.01). Interestingly, CD34+ cell priming was comparable to the addition of high dose IL-2 (at 1000 U/ml) that induced a lysis of 49±3% at a E/T ratio of 10:1 (n=4) (p=0.22). NK cells cultured with CD34+ cells produced greater amounts of IFNγ and TNFa as compared to monocytes (500 pg/mL and 125 pg/mL vs 50 pg/mL and less than 5 pg/mL). CD34+-induced NK activation was prevented by anti-NKG2D antibody (mean lysis with anti-NKG2D was 10±9% as compared to 46±12% with CD34+ cells (p=0.02, n=3) whereas a blocking anti IL-2 antibody had no effect, suggesting that NK cell activation by CD34+ cells involved signaling through NKG2D. This was further confirmed by the observation that NKG2D was downregulated upon stimulation by CD34+ cells (from >80% before culture to <10%
after coculture with CD34+ cells, n=2 experiments). Furthermore, inhibition of lysis was not due to the persistence of anti–NKG2D antibody on the surface of NK cells as shown by the lack of binding of goat anti mouse IgG on NK cells that had been cultured with anti–NKG2D. We then evaluated the expression of NKG2D ligands (MICA and B and ULBP-1, -2 and -3) on the surface of CD34+ cells before and after culture with either TNFα, GM-CSF and TNFα or purified NK cells for 48–72 hrs. Neither MICA/B nor ULBP molecules were detected on fresh CD34+ cells. Culture with TNFα, with or without GM-CSF, was not associated with expression of either NKG2D ligand, whereas purified NK cells induced significant expression of MICA/B (mean MFI 11+7, n= 3 experiments) but not ULBP (MFI consistently <1) molecules. Finally, expression of the costimulatory molecules CD86 and CD40 was greatly increase on CD34 + cells following coculture with NK cells (MFI 14 and 5, respectively) as compared to TNFα, with or without GM–CSF (MFI of <2 and 4, respectively, for CD86 and CD40). Therefore our data suggest the occurrence of MIC-NKG2D mediated bidirectional cross talk between CD34+ cells and NK cells leading to NK cell activation and enhancement of CD34+ ability to stimulate both T and NK lymphocytes.

**CO-11 EXTRACELLULAR NUCLEOTIDES ARE POTENT STIMULATORS OF HUMAN HEMATOPOIETIC STEM CELLS IN VITRO AND IN VIVO**

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Although extracellular nucleotides support a wide range of biological responses of mature blood cells, little is known on their effect on blood cell progenitor cells. In this study, we assessed whether receptors for extracellular nucleotides (P2 receptors, P2R) are expressed on human hematopoietic stem cells (HSC), and whether activation by their natural ligands, ATP and UTP, induces HSC proliferation in vitro and in vivo. Our results demonstrated that CD34+ HSC express functional P2XR and P2YR of several subtypes. Furthermore, stimulation of CD34+ cells with extracellular nucleotides caused a fast release of Ca2+ from intracellular stores and an increase in ion fluxes across the plasma membrane. Functionally, ATP and, to a higher extent, UTP acted as potent early-acting growth factors for HSC, in vitro, as they strongly enhanced the stimulatory activity of several cytokines on clonogenic CD34+ and lineage negative CD34+ progenitors and expanded more primitive CD34+ –derived long-term culture-initiating cells. Furthermore, xenogenic transplant studies showed that short-term pre-incubation with UTP significantly expanded the number of marrow repopulating HSC in nonobese diabetic/severe combined immunodeficiency mice. Our data suggest that extracellular nucleotides may provide a novel and powerful tool to modulate HSC functions.

**CO-12 BONE MARROW- DERIVED PROGENITOR CELL MOBILIZATION COMBINED WITH REVASCULARIZATION SURGERY: AN INNOVATIVE APPROACH FOR PATIENTS WITH POSTINFARCTION CHRONIC HEART FAILURE**

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Recent experimental observations in animal models support the concept that bone marrow derived early progenitors might concur to myocyte and/or endothelial cell regeneration when implanted into the myocardium. This has stimulated an emerging interest on the use of bone marrow cells (BMCs) to repair damaged myocardium. Some pilot studies have been already performed in humans with promising though preliminary results. The mechanism of action of BMCs injected intra-myocardium is still obscure, although induction of neoangiogenesis is the most likely beneficial effect. Basically, the described procedures include BMC collection, ex vivo manipulation and intramyocardial or intracoronary cell infusion. In order to simplify the approach, we have designed an innovative procedure combining BMCs mobilization and revascularization surgery. In our program, mobilization of hematopoietic progenitors was induced by G-CSF + GM-CSF administration. A surgical intervention of transmyocardial scarification was associated, with the aim to favor cardiac colonization of circulating BMCs. Indeed, tissue injury or local flogosis seem critical in promoting homing and development of the donor cells into non-hematopoietic lineages. The surgical procedure of transmyocardial needle punctures described by Sen several years ago is particularly suitable for this purpose. Thus, a pilot study was planned to evaluate
feasibility and efficacy of combining BM-derived progenitor cell mobilization with surgical revascularization according to Sen technique. Preliminary results obtained in 8 patients with end-stage cardiac failure are here summarized. Eligibility criteria included: i. age > 18 and < 80 yrs.; ii. coronary artery disease and severe cardiac failure, with Left Ventricular Ejection Fraction (LVEF) less than 35%; iii. non eligibility to cardiac transplantation; in addition, patients had to be candidate to a coronary artery bypass graft (CABG) or to any other cardiac surgery procedure. Mobilization was induced by the combined administration of G-CSF 5 mcgr/kg s. c. b. i. d. + GM-CSF 2.5 mcgr/kg s. c. /day for 4 to 6 consecutive days. Surgery was planned at day 4 or 5, according to the level of circulating progenitors. The study has been approved by the local Ethical Committee and all patients gave a written informed consent before entering the study program. So far, eight patients (6 males, 2 females; median age: 65 yrs, range: 46-75) with refractory, end-stage heart failure received the combined procedure. All patients had had one or more previous myocardial infarction. The surgical procedure included a CABG (7 patients) or a mitral valve replacement (1 patient), associated with transmyocardial needle punctures in ungraftable fibrotic areas. The areas of scarification were identified according to the scintigraphic and echocardiographic finding of death myocardium not suitable for CABG. Preoperative mobilization induced an increase in the amount of circulating hematopoietic progenitors in all patients. Maximal values of circulating progenitors were recorded at day 4 of cytokine administration, with a median peak value of 20 CD34+ve cells/microl (range: 9-67). No death occurred in the operative period (0-30 days) and all patients were discharged from the hospital. One patient had a non-fatal ischemic stroke few days after discharge due to cerebral embolism secondary to intraventricular thrombus; in addition, 2 late sudden deaths occurred at 1 and 3 months since the procedure. At a median follow-up of 16 mos. from surgery, 6 patients are alive, 5 of them with marked improvement in their LVEF. Radionuclide scan examination showed improvements in each evaluated regional seat including the ungrafted areas. The study demonstrates the feasibility of an innovative procedure, combining BM-derived cell mobilization and transmyocardial scarification. Preliminary results are promising in terms of improvement of the myocardial function. A prolonged follow up in a large series of patients is required to define the efficacy of the procedure.

**CO-13**

INTERLEUKIN-21 SYNERGIZES WITH INTERLEUKIN-15 IN PROMOTING THE EXPANSION AND NK-CELL DIFFERENTIATION OF CD34-LINEAGE HEMATOPOIETIC STEM CELLS

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Hematopoietic stem cells (HSCs) can be operationally defined by their ability to give rise to precursors for the different hematopoietic lineages. Recently, attention has been focused on the existence of HSCs within the heterogenous compartment of CD34-lineage- cells. The common γ-chain-signalling cytokines interleukin (IL)-15 and IL-21 can direct the transition from CD34+ HSCs to NK-cell precursors (NKP). Previously, we demonstrated that highly purified CD34(−)-lineage(−) HSCs from umbilical cord blood (UCB) differentiate towards the lymphoid/NK-cell lineage after in vitro exposure to IL-15 in the presence of a stromal cell feeder layer (Rutella S. J Immunol 2003;171:2977). In the present study, we assessed the effects of the closely related cytokine IL-21 on NK-cell maturation from UCB CD34(−)-lineage(−) HSCs maintained in liquid culture for up to 50 days. Highly purified CD34+CD7−-lineage(−) HSCs were cultured with SCF (20 ng/mL) + Flt-3L (20 ng/ml) either in the presence or in the absence of either IL-21 (20 ng/mL) or IL-15 (50 ng/mL) or the combination of both cytokines. The acquisition of non-MHC restricted lytic activity against tumour cell targets was evaluated using NK-sensitive (K562 cells) or NK-resistant (Raji cells) cell lines that were co-cultured with graded numbers of cytokine-differentiated HSCs. IL-21 potentiated the IL-15-induced cell expansion but exerted minimal effects when used alone. By day +30 of culture, CD34(−)-lineage(−) HSCs were expanded 49.04-fold by IL-21 and IL-15 in combination, compared with 28.48-fold in cultures performed with IL-15 alone and with 2.8-fold in cultures maintained with IL-21 alone (Table 1). Interestingly, exogenous TGF-β at 5 ng/mL significantly inhibited the cytokine-driven proliferation of CD34(−)-lineage(−) HSCs (Table 1), in accordance with previous data on the role of TGF-β in the maintenance of HSC quiescence (Pierelli L. Blood 2000;95:3001). When compared with cells exposed to IL-15 or IL-21 alone, HSCs primed with IL-21 and IL-15 significantly upregulated the expression levels of the T-cell associated antigen CD7, both in terms of percent positive cells and in terms of flu-
MafB over-expression promotes monocyctic differentiation of CD34+ hematopoietic stem/progenitor cells

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A large number of reports indicate that commitment of hematopoietic stem cells (HSCs), along precise differentiation lineages, is the consequence of spontaneous or exogenously induced up-regulation of specific transcription factors. \(^1\) \(^2\) Recent studies have demonstrated the capacity of MafB transcription factor to promote monocyctic differentiation of Myb-Ets transformed avian hematopoietic progenitors. \(^2\) MafB is a member of the Maf basic region/leucine zipper transcription factor family and is characterized by a common carboxy-terminal portion bearing a basic region/leucine zipper domain mediating DNA binding and homo- and hetero-dimer assembly and by an amino-terminal transactivation domain. All Maf family members display binding activity to a common DNA sequence (MARE, Maf Recognition Element), and can either activate or repress transcription depending on the interacting dimerization partner. \(^4\)

In spite of the evidence that MafB behaves as a determinant of monocyctic commitment in transformed avian hematopoietic progenitors, a clear demonstration of the possible role played by this transcription factor in human normal monocytopoiesis is still to be established. Based on this premise the MafB full length cDNA sequence was cloned in the LVIDETAN retroviral vector upstream the Internal Ribosomal Entry Site (IRES) allowing its expression in the context of a bi-cistronic transcript coding for a truncated version of low affinity nerve growth factor receptor (DELTAlNGFR), currently used as reporter gene for hematopoietic cells. The obtained retroviral vector, named LMaf-BFLIDETAN, was used to transduce U937 and THP1 human monocytic cell lines, and human cord blood (hCB) CD34+ hematopoietic stem/progenitor cells.

The extent of mono-macrophagic differentiation was subsequently monitored by morphological and immunophenotypic analysis. To characterize the genetic program induced by MafB over-expression we also assessed mRNA expression profile in transduced U937 by means of the Affymetrix microarray approach. Preliminary results evidenced that retroviral mediated MafB expression lead to induction monocyte differentiation antigens (CD14 and CD11b) in U937 and THP1 cells. In addition, morphological analysis performed by MGG staining on MafB transduced U937 showed that these cells assume a monoblast-promocytic morphology. Microarray analysis, performed by comparing transduced to untransduced U937 cells, indicated that MafB transcription factor hyper-expression lead to induction typical differentiation and activation mono-macrophage markers. To verify these data, we purified hCB CD34+ stem/progenitor cells, transduced them with the analyzed retroviral vector using a retronectin-assisted protocol, and analyzed the extent of mono-macrophage differentiation by immunophenotypic and morphological analysis. At day 7 of culture cells transduced with the LMaf-BFLIDN retroviral vector exhibited a 55±5% expression of CD14 monocye-specific antigen versus
13±1% of untransduced NGFR negative control cells. MGG staining of transduced / purified CD34+, performed at day 14 of culture, displayed a clear macrophagic morphology as compared to the untransduced fraction mainly characterized by elements belonging to the granulocyte differentiation lineage.

References


CO-15

INCUBATION OF MURINE ERYTHROLEUKEMIA CELLS IN SEVERE HYPOXIA INDUCES MASSIVE APOPTOSIS PARALLELLED BY AKT AND ERK5 CLEAVAGE

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We previously showed that severe hypoxia (0.1% O2) favours the self renewal of murine and human normal haematopoietic stem cell. The importance of hypoxia in the regulation of neoplastic stem cells also recently emerged. This study was undertaken to characterize the effects of hypoxia on a murine erythroleukaemia cell line. To this purpose, Friend erythroleukaemia cells were incubated in severe hypoxia (0.1% O2) or normoxia (20% O2) for 7 days; cells incubated in hypoxia (LC1) were then transferred to normoxia (LC2), to determine their potential for overall cell number expansion. The colony-formation efficiency of day-7 hypoxic cultures was unreduced when compared to that of normoxic cultures; however, the incubation in hypoxia during LC1 reduced cell proliferation rate after transfer to normoxia (LC2). The effects of hypoxia at different incubation times were determined with respect to cell cycle and viability. Total cell number was found strongly reduced after 3 days of incubation in hypoxia when compared to normoxia. The Annexin-V test showed that hypoxia doubled the percentage of cells in early as well as late apoptosis. At the end of LC1 (day-3) almost all cells were in late apoptosis, while surviving cells (2%) were in a quiescent state (G0-G1 phase of cell cycle), as demonstrated by flow cytometry. Several molecular parameters were investigated in hypoxic cultures. Hypoxia was found to interfere with the AKT and ERK5 signalling systems. AKT cleavage, as determined by AKT disappearance and appearance of 40-44 kDa AKT fragments, was marked at day 3 of incubation in hypoxia, to increase significantly thereafter. Active (threonine/tyrosine phosphorylated) ERK5 was markedly reduced at day 3 in hypoxia, to disappear at day 6. On the other hand, the expression itself of ERK5 was significantly reduced already after a 1-day incubation in hypoxia; the downmodulation of ERK5 was paralleled by the appearance of a cleaved 30 kDa ERK5 fragment. Under the same conditions, the amount of ERK1/2 in hypoxia was unchanged. These results suggest that AKT and ERK5 are pro-survival signals in these cells and are specifically cleaved in hypoxia-induced apoptosis. The effects of hypoxia on histone acetylation were also determined. We observed that histone H4 was hyp-acetylated at day 3, suggesting that incubation in hypoxia interferes with transcriptional regulation.

CO-16

INVolVEMENT OF THE UROKINASE-TYPE PLASMINOGEN ACTIVATOR RECEPTOR IN HEMATOPOIETIC STEM CELL MOBILIZATION

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Granulocyte colony-stimulating factor (G-CSF), as single agent or in combination with cytokotoxic drugs, is widely used in clinical transplantation to induce hematopoietic stem cells (HSC) mobilization into peripheral blood. Recently, some reports have shown the involvement of the urokinase-mediated plasminogenesis activation system and, in particular, of the urokinase-type plasminogen activator (uPAR) receptor in cell migration and adhesion. We investigated the involvement of uPAR in G-CSF-induced mobilization of CD34+ HSC from 16 healthy donors. The analysis of peripheral blood mononuclear cells (PBMC) showed increased uPAR expression after the G-CSF treatment in CD34+ myeloid and CD14+ monocyte cells, whereas the mobilized CD34+ HSC
remained uPAR-negative. Western blot analysis with a polyclonal anti-uPAR antibody confirmed a progressive increase of uPAR expression in all donors during G-CSF stimulation and showed that PBMC expressed only the intact form of uPAR. G-CSF treatment also induced increased serum levels of soluble uPAR (suPAR). In almost all cases, cell surface uPAR expression on CD33+ and CD14+ cells and serum suPAR levels increased to the maximum extent at days 3-5 of G-CSF stimulations, when CD34+ HSC also peaked into the circulation. Western blot analysis showed that after G-CSF treatment there was not only increase of the intact form of suPAR, but also appearance or strong increase of cleaved forms of suPAR (c-suPAR) in all analyzed sera. c-suPAR was able to chemoattract CD34+ KG1 leukemia cells and bone marrow (BM) CD34+ HSC, as documented by in vitro migratory response of these cells toward a chemotactic suPAR-derived peptide (uPAR84-95). uPAR84-95 induced CD34+ KG1 and BM CD34+ HSC migration by activating the high-affinity formylmethyl peptide (fMLP) receptor (FPR). In addition, uPAR84-95 inhibited CD34+ KG1 and CD34+ HSC migration toward the stromal derived factor 1 (SDF1), thus suggesting a heterologous desensitization of its receptor CXCR4. Finally, we studied the effect of uPAR84-95 on the output of clonogenic progenitors from long-term culture (LTC) of highly purified BM CD34+ cells from normal donors. Non-adherent cells, weekly removed after a 2 h treatment with uPAR84-95, yielded a significant higher number of clonogenic progenitors as compared to those obtained from non-adherent cells of LTC treated with the scrambled version of uPAR84-95. All together, our data document that G-CSF-induced up-regulation of uPAR on circulating CD33+ and CD14+ cells is associated with increased suPAR shedding, which leads to the appearance of serum c-suPAR. c-suPAR could contribute to HSC mobilization by promoting their FPR-mediated migration and by inducing CXCR4 desensitization. Our findings suggest a potential utility of the cleaved form of suPAR, or its derived chemotactic peptide, in the strategies to optimize HSC mobilization, especially in G-CSF poor mobilizers.

Oral Communications
NON-MALIGNANT HEMATOLOGY

CO-17
AN ASSOCIATION OF PLATELET GLYCOPROTEIN GENE HAPLOTYPES AND BLEEDING SEVERITY IN PATIENTS WITH PREVIOUSLY DIAGNOSED VON WILLEBRAND DISEASE (VWD) TYPE 1: RESULTS OF A PILOT STUDY IN 14 ITALIAN FAMILIES

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Von Willebrand Disease (VWD) type 1 is difficult to diagnose because of bleeding variability and low heritability of Von Willebrand Factor (VWF) levels. Secondary gene effects that increase risk for bleeding may modify the phenotype, and platelet adhesion receptors are prime candidates. We compared a bleeding severity score and bleeding times to candidate gene haplotypes within pedigrees of fourteen index cases of previously diagnosed VWD type 1, using a variance component model. The 14 families from Milan have been already enrolled the European study entitled Molecular and Clinical Markers for Diagnosis and Management of Type 1 von Willebrand Disease (Scientific coordinator: I. R. Peake). VWD type 1 patients were classified according to the previous definitions of the Scientific Standardization Committee (SSC) on VWF of the International Society on Thrombosis and Haemostasis (ISTH) such as individuals characterized by reduced levels of normal VWF and positive personal-family bleeding history. A bleeding history was derived from detailed questionnaires administered to all the affected and non-affected members (including index cases), and the severity of bleeding was ranked from 0 to 3 in each of 11 bleeding categories. Donors were genotyped using primer extension method, and nine candidate genes, GP1BA, ITGB3, VWF, FGB, IL6 or TXA2R. Association of platelet glycoprotein gene haplotypes and bleeding severity in patients with previously diagnosed von Willebrand Disease (VWD) type 1: results of a pilot study in 14 Italian families.
association between expression of the VWF promoter haplotype1 and increased levels of plasma VWF:Ag (p=0.0448) and VWF:RCo (=0.024) was also observed. These results establish that genetic differences in integrin subunits α2 and αIIb as well as GPVI can influence the phenotype of previously diagnosed VWD type1.

CO-18
IN VITRO EXPRESSION STUDY OF A NEW MUTATION (R1308L) FOUND IN A FAMILY WITH TYPE 2B VON WILLEBRAND DISEASE CHARACTERIZED BY ALL SET OF MULTIMERS IN PLASMA AND NO THROMBOCYTOPENIA AFTER DESMOPRESSIN
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Type 2B von Willebrand disease (VWD) is characterized by enhanced ristocetin-induced platelet agglutination (RIPA) and by loss of high molecular weight multimers (HMWM) in plasma. A novel 2B von Willebrand factor (VWF) variant (4173G →T, R1308L) was found in four patients with RIPA (0.3–0.4 mg/mL), VWF:Ag (27–51 U/dL), VWF:RCo (23–49 U/dL), bleeding time (6–11 min), all set of multimers in plasma and normal platelet count. Interestingly, a common VWD type 2B mutation, R1308C is characterized by loss of HMWM, low platelet count and RIPA (0.7–0.8 mg/mL), VWF:Ag (26–48 U/dL), VWF:RCo (13–39 U/dL), bleeding time (10–27 min). Mutated rVWF-R1308L and rVWF-R1308C, were transiently expressed in Cos 7 cells, and analyzed for their ability to bind GpIb receptor along with rVWF-WT. Binding of rVWFs to the GpIb platelet receptor was tested by an ELISA method (Federici et al. Haematologica 89:77, 2004), at increasing concentrations of ristocetin (0, 0.125, 0.25 and 0.5 mg/mL), and the rVWF bound to GpIb was revealed by anti-VWF Antibody-HRP reading O. D. 492 nm. The three rVWFs have a similar VWF:Ag and a full set of multimers. Our data demonstrated that the new mutation (R1308L) can enhance the VWF capacity to bind to GpIb receptor, but with lower efficiency compared to the R1308C variant. This might explain why these patients have normal VWF multimers in plasma. The increased binding capacity of VWF–R1308L for GpIb does not cause its spontaneous interaction with platelets, or at least, not as efficiently as VWF–R1308C does. The enhanced RIPA of 0.4 mg/mL, observed in patients with VWF–R1308L, versus 0.7 mg/mL of VWF–R1308C is probably due to the presence of all set of multimers, that strongly compensate for the lower binding capacity to GpIb of this new variant.

CO-19
A FUNCTIONAL SINGLE NUCLEOTIDE POLYMORPHISM IN THE THROMBIN-ACTIVABLE FIBRINOLYSIS INHIBITOR (TAFI) GENE IN ITALIAN CENTENARIANS
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We have previously described in centenarians laboratory signs of marked hypercoagulability, secondary hyperfibrinolysis, high level of VWF, marker of endothelial perturbation, and significantly high frequency of the high risk 4G allele of the PAI-1 -675 polymorphism, mutant factor V and prothrombin gene G20210A mutation. TAFI is a recently discovered glycoprotein combining coagulation and fibrinolysis, TAFI can be activated by thrombin and once activated potently attenuates fibrinolysis. The PRIME prospective study recently shows that an increase in TAFI plasma levels at baseline was associated with a higher incidence of angina pectoris. Recently a twins study on heritability of the prethrombotic state demonstrated that TAFI has a very strong genetic component (82%). In the coding region of TAFI the Thr325Ile variation (1040C/T) has been described. Ile at position 325 confers stability to TAFIa increasing the antifibrinolytic potential. Purpose of the study. To evaluate the incidence of TAFI 325Ile/Ile genotype in centenarians selected with established enrollment criteria of the Italian Centenarians Study and in a group of normal subjects randomly selected from the same metropolitan communities. Materials and Methods. 100 centenarians (100–106 yrs) and 100 controls (25–62 yrs) were the cases. The TAFI antigen levels were measured by ELISA. The identification of TAFI genotype was carried out by the polymerase chain reaction. The PCR products were digested using the Spel endonuclease as described by Brouwers et al. Results. The levels of TAFI Ag were not different in centenarians and in the controls (103± 50 vs 110±35). The Ile325 variant (T/T and C/T) results in lower TAFI plasma levels. A significant difference was detected in the prevalence of genotypes between centenarians and controls. The T/T genotype is more frequent in centenarians than in controls: 18% vs 7% (p=0.05). Thr325Ile polymorphism is significantly correlated with TAFI Ag levels with C/C genotype corresponding with the highest and the T/T genotype with the

haematologica vol. 89(suppl. n. 6):september 2004
lowest TAFI Ag levels (centenarians: r=0.62 p= 0.001; controls: r=0.82 p= 0.001). Conclusions. Centenarians have an increased amount of thrombin generated by different pathway, factor VII–tissue factor cascade and by factor XIIa activation and a progressive fibrinolysis expressed by very high levels of D–dimer and plasmin–antiplasmin complex (Mari et al Blood 1995, Coppola et al Blood Coagulation and Fibrinolysis 1996). Centenarians have increasing frequency of the T/T genotype, leading to downregulation of fibrinolysis which may contribute to the paradox of this peculiar population. However the possession of several high–risk alleles and well–known atherothrombotic risk markers appears to be compatible with longevity and/or health.

**CO-20**

**NEW PROGNOSTIC FACTORS IN THE STUDY OF THROMBOTIC THROMBOCYTOPENIC PURPURA**

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Thrombotic thrombocytopenic purpura is a rare disease described for the first time in 1924 by Moschowitz. This disorder occurs with an annual incidence of 1 case for 10⁶ residents and 2.7 cases for 10⁵ according to the TTP HUS Oklahoma Register data. In Sardinia, evaluating the 40 cases presented at the U. O Haematology of Cagliari between 1992 and 2003, we have recorded an incidence strongly superior than that reported in literature with 8.5 case for 10⁶/year. The factor that initiates the disease seems to be endothelial damage, resulting in the aggregation of platelets into the micro vessels and in the occlusion of terminal arterioles and capillaries. The histopathologic pattern of TTP shows endothelial cell and detached from the basal membrane, which is then covered by platelet rich thrombi and platelets aggregates. Platelets aggregation derives from the presence of unusually Large multimeric forms of vWF in the plasma of patients with TTP. The various isoforms of ADAMTS 13 (a disintegrin and metalloprotease with thrombospondin type 1 domains 13) gene have biological functions further than the proteolytic activity of vWF. Mutations on ADAMTS 13 gene are reported in familial TTP; 65,5% of patients with an activity of ADAMTS13 inferior to 5% present antibodies against the vWF cleaving protease. These data suggest that the plasmatic activity of the vWF CP is an efficient prognostic factor such as the measurement of the Antigen level and both depend on the haemostatic conditions of patients. Polymorphisms are mutations of DNA that occur with a frequency of 1%; 90% of polymorphisms derive from the substitution of a single base (SNP). Some of themes play a role in the gene expression regulation or in the coded protein function. Other of them is relevant to determine the resistance to treatment. Aim of this study is searching the presence of polymorphism into the metalloprotease genes. It is important to evaluate the frequency of the presence of the ADAMTS 13 gene polymorphism, in particular of the mutant P475S, the most common SNP associated with variation of the activity of the vWF–CP. Studying the metalloprotease MMP1; 55,5% of patients with TTP in complete remission has show an eutyrozygosis 1G/2G; 44,4% an omozygosis 1G/1G; none 2G/2G. The polymorphisms frequency (1G/2G, 1G/1G) recorded are significantly different if compared with a Sardinian population of 176 individuals random. It could be interesting to find an association between the polymorphisms of the metalloprotease genes and the risk of disease or the resistance to treatment. It is important to evaluate the expression of ADAMTS 13, using the RT–PCR, and its activity, the positivity of the mutant P475S, the correlation between genic polymorphisms and the individual susceptibility to the TTP, the quantity of vWF in blood samples and of UL vWF(unusually large multimeric forms of vWF) and the quantity of their proteases. These new molecular techniques give us the possibility to monitories the patient from the exordium of the disease, after the plasma–apheresis cycles, after therapy and during the follow-up. Knowing the genetic aspects that may condition the individual response to therapy and the familial correlations could give us the possibility to make more appropriate therapeutic decisions with a significant benefit for the patient and also with a relevant

**CO-21**

**TISSUE FACTOR AND ANGIOGENESIS ARE SIMULTANEOUSLY INHIBITED BY ALL-TRANS RETINOIC ACID (ATRA) IN ENDOTHELIAL CELL STIMULATED BY ACUTE PROMYELOCYTIC LEUKEMIA CELLS**

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Remission induction of human APL by ATRA parallels the rapid resolution of the associated coagulopathy. This is because ATRA is able to modulate the hemostatic system, including the inhibition of Tissue Factor (TF) expression by endothelial cells. TF, the main activator of blood coagulation, is involved in tumor angiogenesis. To understand whether ATRA

**VIII Congress of the Italian Society of Experimental Hematology, Pavia, September 14-16, 2004**
modulation of endothelial TF is associated to the occurrence of antiangiogenic effects, in this study we evaluated whether ATRA may affect both TF expression and new capillary-like tube formation by microvascular endothelial cells (HMEC-1) stimulated by leukaemic cell (APL cell NB4). HMEC-1 were incubated with NB4 conditioned medium (CM), in the presence of increasing concentrations of ATRA (0.0001–1.0 μM) or vehicle (control), then TF (activity and antigen) and the capillary-like tube formation (by the Matrigel model) were evaluated. Also the effect of ATRA on angiogenesis induced by purified proangiogenic factors (i.e. VEGF and bFGF) was characterized. Capillary-like tube formation was examined under phase-contrast microscopy and tube length determined by image analysis software. Control CM induced a modest tube formation (total tube length = 330 mm/cm²), which was dose-dependently decreased by ATRA, with a 36% inhibition by 1.0 μM ATRA (p<0.01). NB4-CM induced a significant (p<0.05) increase in total tube length (40% increment) and significantly (p<0.01) increased the expression of TF. The addition of ATRA significantly counteracted the increase in TF expression and completely inhibited the induction of angiogenesis by NB4-CM (100% inhibition). Cytokine measurement of the NB4-CM revealed the following content: VEGF> IL-1β> TNF-α> b-FGF. Inhibition studies with neutralizing MoAbs against proangiogenic factors demonstrated that a combination of anti-VEGF + anti-TNFα MoAbs was necessary to 100% inhibit NB4 cell proangiogenic action. In addition, ATRA completely inhibited tube formation induced by standard purified VEGF, b-FGF, and VEGF + b-FGF combination. In conclusion, in this experimental system, ATRA inhibition of endothelial cell TF was coupled with angiogenesis downregulation. Whether or not the two phenomena are interdependent remains to be elucidated. Inflammatory cytokines also played a role in our system, which points to ATRA as a valuable tool to identify pathways linking thrombosis, inflammation, and cancer.

**CO-22**

**IN VITRO DIFFERENTIATION OF ENDOTHELIAL CELLS FROM HUMAN CD133+ CELLS**


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Recent findings support the hypothesis that bone marrow and peripheral blood contain endothelial progenitor cells. The molecular mechanisms responsible for vasculogenesis and angiogenesis are not completely understood. Several growth factors are involved in regulation of endothelial proliferation and differentiation. In this study we report that CD133+ cells from human normal bone marrow and granulocyte colony-stimulating factor-mediated peripheral blood can be induced to differentiate in vitro into endothelial cells in defined culture conditions. Isolated CD133+ cells were grown on fibronectin-coated plates in different media with or without fetal bovine or horse serum with the addition of different combination of growth factors. The CD133+ cell fraction express CD34, DR, CD117, CD105, CD31, but did not express VE-cadherin or vWF. CD133+ cells give rise to hematopoietic progenitors, LTC-IC plated in LDA and endothelial colonies in collagen based media. When cultured in the presence of serum, VEGF, FGF-β, IGF-1, TPO, IL6, IL3, SCF, Flt3-lig, CD133+ cells generate both adherent and proliferating nonadherent cells. Phenotypic analysis of the cells within the adherent population reveals 70% CD45− cells, no CD14+ cells, 20–25% CD31+ cells, 5–15% CD105, CD34−, VE-cadherin+ and vWF+ cells. Nonadherent cells, 90% CD45+ cells, give rise to both hematopoietic and endothelial colonies. We showed that, depending on culture conditions, CD133+ cells can be differentiated along the endothelial or the hematopoietic pathway. We don’t know if CD133 cell population contains separate progenitors for endothelial and hematopoietic cells or a common precursor even if several different lines of investigation suggest the presence of functional hemangioblastic activity in the adult.

**CO-23**

**MITOCHONDRIAL FERRITIN EXPRESSION IS AN EARLY EVENT IN SIDEROBLASTIC ERYTHROPOIESIS**

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We have recently reported an unusual intronless gene on chromosome 5q23.1 encoding a 242-AA precursor of a ferritin H-like protein. This 30-kDa protein is targeted to mitochondria and processed to a 22-kDa subunit that assembles into typical ferritin shells and has ferroxidase activity. This new mitochondrial ferritin (MtF) may play an important...
role in regulating mitochondrial iron homeostasis and heme synthesis. A mitochondrial accumulation of MtF has been demonstrated in the erythroblasts of refractory anemia with ring sideroblasts (RARS) but not in refractory anemia (RA). On the other hand, some of us have shown that in low-risk myelodysplastic syndromes (MDS) the enhanced apoptosis of early erythroblasts is mediated through a constitutive cytochrome c release from mitochondria, and that G-CSF strongly inhibits this process. Since preliminary findings indicate that cells overexpressing MtF are more resistant to oxidative damage and also to the apoptotic signals, we assessed the kinetics of MtF accumulation during erythroid maturation in MDS in relation to cytochrome c release, as well as the effect of G-CSF. Our aim was to evaluate at which stage of erythroblast differentiation mitochondria accumulate MtF in RARS and whether MtF accumulation reflects a defense mechanism of the cell. CD34 positive cells from the marrow of 9 patients with MDS (5 RARS and 4 RA) and from 3 healthy donors were cultured for 14 days using a liquid-culture procedure, according to previous publications. Samples of cultured cells were removed at days 4, 7, 11 and 14, cytto centrifuged on glass slides, and analyzed for the distribution of MtF and of cytoplasmic H ferritin (HF) using immunocytochemical methods. Translocation of cytochrome c from mitochondria to cytosol was determined by immunofluorescence. Freshly separated CD34 positive cells from all subjects were negative for MtF. MtF was barely detectable in few cells from normal samples (0–4%), day 4–14), and RA erythroblasts showed a median of 3% (0–8%) positive cells. The contrary, RARS erythroblasts showed an early increase in MtF positive cells and a continuous increase during the culture period (day 4: 10%, day 7: 17%, day 11: 13%, day 14: 19%). There was no correlation between MtF expression and cytochrome c release except at day 14. Interestingly, G-CSF significantly reduced the cytochrome c release in RARS and to a more moderate extent in RA, and tended to increase MtF expression in some RARS erythroblast cultures. HF expression was variable and not influenced by G-CSF. In MDS it was higher than in normal samples; in RARS the percentages of positive cells tended to diminish at late stages. In conclusion, our findings show that the expression of MtF occurs very early during RARS erythroid differentiation, in cells that are still CD34 positive and without any visible iron accumulation. Iron accumulation, probably as a consequence of a primary defect in iron metabolism, may cause further damage to the mitochondria and result in cytochrome c release and apoptosis. Alternative mechanisms, not associated with iron-overloading, may be involved in RA pathogenesis, since these cells show cytochrome c release but very moderate MtF expression. Interestingly, G-CSF treatment seems to inhibit apoptosis by upregulating compensatory mechanisms for cell survival rather than affecting mitochondrial iron metabolism.

**CO-24**

**ANALYSIS OF CD8+ CD57+ LYMPHOCYTES IN PATIENTS WITH PAROXYSMAL NOCTURNAL HEMOGLOBINURIA**

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Paroxysmal nocturnal hemoglobinuria (PNH) is an acquired clonal disorder of the hematopoietic stem cell (HSC) characterized by intravascular hemolysis, venous thrombosis, and variable degrees of bone marrow failure. In PNH a somatic mutation of the X-linked PIg-A gene in HSC results in complete or partial deficiency of all proteins anchored by the glycosylphosphatidylinositol (GPI) on the membrane of the mutated HSC and in its mature progeny. The close association between PNH and Idiopathic Aplastic Anemia, and numerous other lines of evidence support the hypothesis that auto-reactive T cells might be the cause of the expansion of the hematopoietic PNH clone(s). Specifically, these T cells might damage selectively normal HSC, whereas PNH HSC survive and expand because they escape the attack. Our recent observation of a unique patient with PNH and with a large granular lymphocyte (LGL) leukemia (Karadimitris et al. , Br J Haematol 2001;115:1010) has strongly suggested the possibility that this clonal expansion of T cells, which have a CD8+ CD57+ phenotype, could be responsible for the damage to normal HSC in this patient. For this reason we have measured systematically the percentage of the CD8bright CD57+ T cells in the peripheral blood of 15 PNH patients and of 18 healthy individuals. The proportion of this cell population was quite variable and very similar in patients (7.4±6.3; range: 0.8–22.3%) and in healthy individuals (6.5±5.2; range: 0.9–1.2. p>0.5). Next, we have investigated the molecular features of these cells. Sorted CD8bright CD57+ T cells were characterized with respect to the size distribution of the complementarity-determining region 3 (CDR3) of the T-cell receptor (TCR) variable (Vp) chain genes. In healthy individuals this analysis yields a ladder of normally distributed bands of different sizes. By contrast, in 13 out of 14 PNH patients this analysis has yielded a markedly non-random (oligoclonal) pattern; and in each patient some clones are predominant. In 6 patients we were able to analyze...
follow-up samples and after 6 months or more the oligoclonal pattern was persistent. Because these sequences may give some clue about the identity of the target molecules of these T-cell clones, we have proceeded to sequencing the TCR-Vβ chain genes in the expanded clones in 7 patients. From each of these patients we have observed an average of 25 different TCR-Vβ CDR3 sequences (out of an average of 80 total sequences obtained). In each patient only one or two sequences were predominant. From a preliminary alignment analysis of the TCR-Vβ sequences we have found an identical TCR-Vβ sequence in two different patients; and in two other patients we have found another, nearly identical sequence. These observations support the hypothesis that the CD8+bright CD57+ T-cell population include a subset of cells that may be directly implicated in the pathogenesis of PNH.

Oral Communications

STEM CELL TRANSPLANTATION

CO-25
CORRELATION BETWEEN PRETRANSPLANT RECIPIENT BLOOD MDC LEVELS AND ACUTE GVHD

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Animal models have suggested that host dendritic cells are essential in the induction of acute GVHD following allogeneic HSCT, through the direct presentation of host alloantigens to donor T lymphocytes. Human DC comprise at least two distinct subsets, i.e. myeloid DC, including their monocytic precursors, that have been associated with the triggering of alloimmune responses, and plasmacytoid DC that have been shown to modulate alloimmune responses, through induction of Th2 or T regulatory activity. Therefore it has been hypothesized that host mDC and pDC may differentially regulate acute GVHD. In this study we employed flow cytometry to enumerate both mDC (lin-, HLA-DR+ and CD11c+) and pDC (lin-, HLA-DR+ and CD123+) in the blood of patients receiving an allogeneic HSCT. CD14+ monocyte numbers were also determined as mDC precursors. Fifty consecutive patients undergoing HSCT from HLA-matched either related (n=28) or unrelated (n=22) donors were enrolled in the study. The stem cell source was bone marrow in all unrelated donors, and G-CSF mobilized PBSC in related donors. Indications to transplant were AML (n=12), ALL (n=11), MM (n=10), CML (n=7), NHL (n=6) and HD (n=4). 13 patients (26%) received reduced dose conditioning regimens (RIC). All patients received CsA and MTX as GVHD prophylaxis. Moreover, 26 patients (52%) received ATG before transplant. mDC and pDC PB counts were significantly lower in patients as compared to 28 age-matched healthy controls [8.8 cells/µL (25th to 75th percentile 3.5-14.5)] mDC, and 2.8 (1.3-5.5) pDC, vs 15.5 (12.1-25.1) and 8.6 (5.6-13.1), respectively] (p<0.001). However the mDC/pDC ratio was significantly higher in the patient group [3.5 (1.6-6.2) vs 1.7 (1.3-2.6), p=0.002], indicating a preferential decrease of pDC. Monocyte counts were not significantly different. mDC, pDC and monocyte counts varied depending on the diagnosis. Among 46 patients evaluable, 12 (26%) developed acute GVHD grade II-IV. Risk factors significantly associated with acute GVHD were older age (p=0.01), PBSC transplants (p=0.02) and the absence of ATG in the conditioning regimen (p=0.01). Patients with acute GVHD had significantly higher pre-transplant mDC:pDC ratio [5.7 (3.3-16.4) vs 3.1 (1.6-5.5)] (p=0.03) and monocyte counts [395 (326-625) vs 284 (187-395)] (p=0.02). To exclude the potentially confounding effect of ATG administration, a subset analysis was performed in PBSC patients, only 3 of whom had received ATG before transplant. Among 26 evaluable patients, 10 (39%) developed acute GVHD grade II-IV. Besides older age (p=0.02), the only risk factors significantly associated with acute GVHD in PBSC patients were the pre-transplant mDC:pDC ratio [5.7 (4.1-13.1) vs 1.7 (1.2-2.9)](p=0.008) and monocyte counts [395 (352-710) vs 259 (199-314)](p=0.004). Patients with a mDC:pDC ratio > 2.3 had a significantly higher probability of developing acute GVHD (61% vs 14%, p=0.05). Pretransplant monocyte counts > 395/µicroL also correlated with a higher incidence of acute GVHD. No correlation was observed between pretransplant recipient PB mDC, pDC or monocyte counts and chronic GVHD. Since our data suggest a correlation between acute GVHD and the balance between mDC (including their monocytic precursors) and pDC in PB, further studies will exploit clinical strategies to deplete or inactive host mDC before transplant as a means of GVHD prophylaxis.
Advances in understanding T-cell function and the human leukocyte antigen (HLA) system have led to the development of strategies for adoptive immunotherapy using donor-derived T cells for treatment of hematological malignancies. A major risk of this approach is the development of graft-versus-host disease (GVHD), which can be minimized by selecting donors whose HLA types are better matched to recipients. However, a major risk of generating alloreactive T cells is the possibility of inducing GVHD. This risk can be reduced by using T cells that are partially matched in their HLA types.

In a recent study, we investigated the possibility of separate T cells that are partially matched. We demonstrated that a large number of T cells can be generated in vitro from partially matched donors. These T cells can be used to treat leukemia by identifying antigen-specific T cells that are able to recognize leukemia blasts (LB). Using both HLA-matched and partially matched donors, we were able to separate T cells that were able to recognize LB. However, these T cells were not able to recognize patient non-leukemia cells.

In order to verify the possibility of separating T cells that recognize an antigen only present or upregulated on LB, we tested these T cells in vitro for their ability to lyse LB and patient non-malignant cells. Some of the anti-leukemia T cell lines displayed a sizeable cytotoxicity against patient non-leukemia cells, even though this alloreactivity was lower than that observed against patient LB. The exact antigen specificity of these CTL lines is unknown, even though this alloreactivity was lower than that observed against patient LB. In this study, we used T cells that were partially matched and were able to mediate GVL effect to these involved in the development of GVHD. This risk could be reduced by using T cells that are partially matched. In future studies, we plan to use T cells that are partially matched and were able to mediate GVL effect to these involved in the development of GVHD. This risk could be reduced by using T cells that are partially matched.
inary results are confirmed in a larger series of patients, one additional donor selection criterion may have been identified for poor-risk AML patients transplanted in chemoresistant relapse.

**CO-28**

**LONG-TERM FOLLOW-UP OF IN VIVO TELOMERE DYNAMICS AND IN VITRO FUNCTIONAL BEHAVIOUR OF HUMAN HEMATOPOIETIC STEM CELLS IN AUTOGRAFTED LYMPHOMA PATIENTS**

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Telomere length is considered a valuable predictor of the replicative capacity of human hematopoietic stem cells. Indeed, a progressive telomere shortening characterizes in vitro growth of hematopoietic cells. However, less is known on the dynamics of telomere shortening in vivo during physiological aging and following a non-physiological replicative stress. To investigate cellular senescence markers of hematopoietic cells exposed to replicative stress induced by bone marrow reconstitution following stem cell autograft. Thus, the study was aimed to evaluate: i. telomere length and ii. in vitro functional characteristics of bone marrow (BM) and peripheral blood (PB) cells obtained from long-term surviving patients previously treated with intensive chemotherapy and autograft. Thirty-two lymphoma patients were examined, at a median time of 73 months (range 42–125) since autograft. All patients had received a high-dose sequential chemotherapy treatment followed by peripheral blood progenitor cell (PBPC) autograft. There were 20 male and 12 female; their median age at autograft was 40 years (range 21–60). A Southern blot procedure using a chemiluminescence-based assay was employed to determine telomere length on samples from grafted material obtained through leukapheresis at the time of maximal PBPC mobilization as well as on BM and PB samples obtained from long-term survivors previously treated with intensive chemotherapy and autograft. Thirty-two lymphoma patients were examined, at a median time of 73 months (range 42–125) since autograft. All patients had received a high-dose sequential chemotherapy treatment followed by peripheral blood progenitor cell (PBPC) autograft. There were 20 male and 12 female; their median age at autograft was 40 years (range 21–60). A Southern blot procedure using a chemiluminescence-based assay was employed to determine telomere length on samples from grafted material obtained through leukapheresis at the time of maximal PBPC mobilization as well as on BM and PB samples obtained at long-term during follow-up. These latter samples were also studied for their in vitro functional characteristics, assessed by short (CFU-GM, BFU-E and CFU-Mix) and long-term culture assays (LTC-IC). All patients were autografted with large quantities of hematopoietic stem cells (median autografted CD34+ve cells/kg: 9,8 x 106, range 2–24). Telomere length was found slightly shortened in BM mononuclear cells from samples taken at follow-up compared to samples from grafted material (median telomere length: 6895 bp, range 4631–8983 vs 7073 bp, range 4991–9230, respectively; p=ns). No marked differences were observed in telomere evaluation between BM and PB cells. PB telomere length of follow-up samples was then compared with telomere length of PB from age-related normal subjects. This allowed to verify that PB telomere status fell within the range of normality in all but one autografted patients. BM and PB samples were also assessed for their in vitro growth characteristics. A slight reduction in committed progenitors was observed; however, median values were still within the normal ranges. On the contrary, the more immature LTC-IC population was definitely reduced, with median values significantly lower than those observed in control normal subjects. The proliferative stress induced by intensive chemotherapy and post-graft hematopoietic reconstitution does not imply marked telomere loss in BM and PB cells at long-term, provided that large quantities of PBPC are used for autografting; ii. stem cells present in the graft or surviving after high-dose therapy are capable of stable reconstitution of the committed progenitor cell population while immature LTC-IC progenitors remain persistently impaired even at up to 10 years since autograft, in spite of the abundant PBPC autografted.

**CO-29**

**REPRODUCIBLE, CLINICAL GRADE GENERATION OF HLA-MATCHED DONOR DERIVED CYTOTOXIC T CELLS AGAINST AML AND ALL BLASTS**

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During the past few years several in vitro-protocols have been described aimed to manipulate donor T
cells in order to improve graft versus leukemia (GvL) while diminishing graft versus host disease (GvHD) potential. However only few adoptive immunotherapy trials have been reported so far, due to the complexity of such approaches. Aims: We evaluated the possibility of generating, expanding and characterising donor derived cytotoxic T cells (CTL) directed toward different types of AML and ALL leukaemia using a rapid, highly reproducible and efficient clinical grade in vitro method. Material and methods: After 5 days of in vitro culture with of Stem Span (Life Technologies, Vancouver, Canada) culture medium, GM-CSF (100 ng/ml, Schering Plough), IL-4 (50 ng/mL, Euro-Clone, U. K.) and calcium ionophore (100 ng/mL, Sigma Aldrich, Milan, Italy), we were able to successfully differentiate all samples of AML (N=13) and B precursor ALL (N=23) into leukemia derived-antigen presenting cells (LD-APC). No differentiation was possible when T-ALL (N=3) were studied. LD-APC showed a mature dendritic cell phenotype (CD40+, CD83+, CD86+ and CD80+), became able to uptake dextran FITC and made cord blood derived naive T cells proliferate. 5 ALL and 4 AML derived APC successfully generated anti leukemia CTL from cord blood and peripheral blood derived naive T cells. Over 14 days of in vitro culture naive T cells derived-antigen presenting cells (LD-APC) from 4 ALL patients were used as antigenic PHA blasts. They were perforine negative, granzyme B positive (median expression was 70%) and that they were able to produce IFNγ upon specific stimulation (median expression was 34%). As a comparison LD-APC from 4 ALL patients were used to generate leukemia reactive CTL from cord blood naive T cells. Over in vitro culture naive T cells changed their phenotype into effector memory cells, became perforine, granzyme and IFN-γ+61543; positive and more interestingly showed a higher cytotoxicity against leukemia blasts as compared to adult CTL (median 45% at E: T ratio 10:1). These experiments were performed under GMP conditions so to provide a protocol for further scaling up. These results demonstrated the feasibility to generate highly cytotoxic, leukemia reactive T cell lines in the majority of AML and ALL patients and provide the biological background to design clinical protocols of adoptive immunotherapy in patients with leukemia relapse after allergic transplantation.

CO-30
SECONDARY MYELODYSPLASTIC SYNDROME/ACUTE LEUKEMIA FOLLOWING HIGH-DOSE CHEMOTHERAPY AND AUTOGRFT: AN ANALYSIS OF RISK-FACTORS ON 307 LYMPHOMA PATIENTS TREATED WITH THE HIGH-DOSE SEQUENTIAL (HDS) CHEMOTHERAPY APPROACH
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High-dose chemotherapy (HDT) with autologous stem cell transplantation is an important therapy for both non-Hodgkin’s Lymphoma (NHL) and Hodgkin’s Lymphoma (HL). Its clinical applicability has been considerably amplified by using peripheral blood progenitor cells (PBPC). Indeed, HDT with autograft is now the most frequently employed treatment for patients <65 y. o. at relapse and is an effective first-line treatment option for patients younger than 60 yrs. with poor prognostic features. Post-chemotherapy secondary myelodysplastic syndrome/acute leukemias (s-MDS/AL) represent an increasingly emerging cause of failure in patients potentially cured after HDT and autograft. The occurrence of post-autograft sMDS/AL has been reported by several studies, with 5-yr actuarial incidence rates quite heterogeneous, ranging between 3 and 18%. In addition, several factors have been associated with the occurrence of sMDS/AL, however conclusive results are still lacking. Since 1988, we have employed the high-dose sequential (HDS) chemotherapy approach with bone marrow (BM) or PBPC autograft, in the management of high-risk lymphoma patients. These patients have been carefully monitored not only for disease recurrence but also for late adverse toxicities including secondary malignancies. Based on this sample a retrospective analysis has been recently performed with two main aims: i. to assess the frequency of sMDS/AL in a large series of patients followed up to 15 yrs. ; ii. to identify risk factors for sMDS/AL in the HDS setting. The study has been performed on 307 lymphoma patients treated with a HDS program at our institution between 1988 and 2003.Our series included 38 patients with HL, and 269 with NHL (153 with high-grade and 116 with
low-grade NHL). Median age was 46 yrs. (range 16–70); there were 180 male and 127 female. Overall, 207 patients received HDS as first-line therapy, and 100 patients as salvage treatment following one or more lines of conventional chemo-radiotherapy. The HDT program included either the original HDS regimen or one of the subsequently developed second- and third-generation schemes, identified as i-HDS (intensified) and C-HDS (Ara-C-supplemented), as described. Among 307 patients entering the HDS protocol, 240 concluded the whole program with the final autograft. Most patients were autografted with PBPC and only a few received either BM cells alone or BM cells combined with PBPC. All patients have been monitored with clinical, laboratory and radiologic reassessments at given intervals during follow-up. At a median follow-up of 5.5 yrs., 134 (65%) of 207 patients receiving front-line HDS are presently alive, while 44 survive in the group of 100 patients treated for refractory/recurrent lymphoma. Overall, among 307 patients receiving a HDS approach, 14 (4.5%) developed s-MDS/AL (10 after PBPC and 3 after BM autografting, 1 after HDS without the autograft procedure). The actuarial projection of developing s-MDS/AL is 4.8% at 5 yrs. Refractory/relapsed status at HDS was the only factor strongly associated with the development of sMDS/AL after BM autografting, 1 after HDS without the autograft procedure. The actuarial projection of developing s-MDS/AL is 4.8% at 5 yrs. Refractory/relapsed status at HDS was the only factor strongly associated with the development of s-MDS/AL.

Although reduced-intensity conditioning (RIC) regimens induce engraftment of allogeneic stem cells with a relatively low toxicity, GVHD remains a significant cause of morbidity and mortality. In vivo T cell depletion with alemtuzumab (100 mg total dose) has been shown to reduce the incidence of GVHD. However, the in vivo persistence of alemtuzumab at lympholytic concentrations impairs the immunereconstitution and limits the efficacy of the graft-versus-tumor (GVT) effect. The purpose of our trial was to test the effect of lower dose of alemtuzumab on GVHD and GVT effect. We have enrolled 26 patients receiving allogeneic stem cell transplantation (SCT) from HLA identical (n=23) or one-antigen mismatched (n=3) family donor. Patients were conditioned with a RIC regimen including thiopeta (10 mg/kg), fludarabine (60 mg/m2) and cyclophosphamide (60 mg/kg). GVHD prophylaxis consisted of short-course MTX and CSA (2 mg/kg). Alemtuzumab was given at 7.5 (n=16) or 15 mg/ms (n=10) on day –2. Patients with progressive disease (PD) or residual disease without GVHD, were considered eligible for donor lymphocyte infusions (DLI). Median age was 57 years (range: 41–65). Diagnosis were: MM (n=11), NHL/HD (n=11), MDS/AML (n=4). All patients received a median of two previous lines of therapies and 54% received a previous autograft. Before SCT, 27% of the patients had chemorefractory disease. Twenty-three patients have a minimum follow-up of 4 months (median follow-up 300 days) and are evaluable for toxicity, immunereconstitution, incidence of acute GVHD, disease response. The estimated 1-year TRM was 11%. All patients had a sustained engraftment. The majority of patients (86%) were full donor (peripheral blood T cells and granulocytes) at day +60 after SCT. Two patients after initial full donor engraftment experienced a loss of T cell engraftment concomitant to relapse of disease. The median value of CD4+ and CD8+ at day +90 were 130/ul and 200/ul. The median value of peripheral dendritic cells (DC) at day +90 were 3.5/ul for DC1 (CD11c+) and 2.9/ul for DC2 (CD11c-). Chimerism analysis on DC1 and DC2 cells has been performed on 10 samples: by day +30 more than 95% of DC were of donor origin. We analyzed TCR (TCR) BV spectratyping in 12 patients: 6 of them, evaluable at 9–12 months after SCT, had a recovery of a complex TCR repertoire. Eleven patients (47%) received DLI for PD (n=6), residual disease (n=2) or for persistence of CMV infection (n=3). The median time for first DLI was 190 days. 37% responded to DLI. The overall incidence of grade II–IV acute GVHD pre- and post-DLI were 9% and 39%, respectively (no grade IV GVHD occurred). The incidence of chronic GVHD pre- and post-DLI were 0% and 17%, respectively. Currently, 21 of 26 patients (80%) are alive: 12 in CR (n=3 molecular remission), 6 in PR and 3 not yet

Related mortality

DECREASED INCIDENCE OF ACUTE GVHD AND TRANSPANT RELATED MORTALITY


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evaluable for response. Five of 26 patients (19%) died from their underlying disease (n=3) or from TRM (n=2). The estimated 1-year progression-free survival and overall survival were 54% (95% CI, 28% to 80%) and 68% (95% CI, 45% to 91%), respectively.

Summary: Our results suggest that: (1) low doses of alemtuzumab reduces the incidence of de novo acute GVHD (probably by a depletion of host DC) and promote a rapid immunereconstitution; (2) the encouraging results in terms of OS suggest that relapsing patients can be rescued with DLI; 3) the role of alemtuzumab in HLA matched sibling allografts deserves therefore further studies.

C0-32
A NOVEL STRATEGY FOR CELL THERAPY OF CHRONIC MYELOID LEUKEMIA USING EX VIVO TREATMENT OF PH POSITIVE CELLS WITH DECITABINE AND WITH THE BY-STANDER TRANSFER OF CD40L


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CML is the prototype of disease that is eradicable in the setting of allogeneic stem cell transplantation (SCT), and the curative response seems to be related not only to chemo-radiotherapy received for conditioning, but also and even more to a specific anti-leukemia immunologic effect, referred as graft versus leukemia (GvL). Here we describe a method for cellular immunotherapy of CML, based upon the induction of expression of PRAME (preferentially expressed antigen of melanoma), a cancer associated antigen which has been shown to be recognized by autologous cytotoxic T cells in the context of MHC-I restriction and on the improvement of the capacity of the leukemic cell to present the tumor antigen. The expression of PRAME is normally very low in Ph positive cells from CML patients at the onset of the disease. Indeed, we analysed bone marrow cells of 10 patients with early chronic phase CML and found that this protein is expressed, at very low titers in only 8 of them. In addition, we incubated the K51, a Ph+ cell line, and Ph+ primitive cells from untreated patients in the presence of scalar amount of IFN (from 10 to 200 U/mL), of imatinib (0,1 to 1,0 micrM), and of hydroxyurea and we found that the more common used drugs in CML did not modify the expression PRAME gene. We also treated in vitro the same Ph positive cells with decitabine, a demethylation agent which is already used in the treatment of myelodysplastic syndromes, given the expression of PRAME is regulated in embryonic cells by methylation status at the CpG islands of the promoter region of this gene. Our findings indicated that decitabine showed a dose-dependent (1 to 5 micrM) effect in the inducing the expression of PRAME. Indeed, we found that after 48 hours of in vitro incubation of Ph+ cells in the presence of decitabine, the level of PRAME specific mRNA increased up to 15 fold respect the untreated control cultures. To increase the capacity of the malignant cells to present this tumor antigen, we used the by-stander technique to induce the expression of CD40L on Ph+ CML cells. The method is based on the co-culture of decitabine-treated Ph+ cells with MCR5 human embryonic fibroblast cell line, previously transduced with a adenoviral construct containing the full-length cDNA for the CD40L. The transduced MRC5 cells express at high level CD40L at their membrane and this latter protein has been shown to have an intrinsic high capacity to transfer to other cells in culture. Indeed, we found that co-culture results in high effective transfer of CD40L on Ph+ cells and cells harvested from the MRC-5 feeder layer had > 70% of expression of CD40L after 48 h of co-culture. Finally, we used ELISpot assay to measure the IFN-γ production from autologous peripheral blood T cell when incubated in the presence of Ph positive cells with by-stander induced expression of CD40L, and verified that the treatment with decitabine alone induces 5-fold increase of IFN-γ, while cells treated with decitabine and with the by-stander transfer of CD40L induced 10-fold increase of IFN-γ production. Our results indicate that this combined in vitro treatment of Ph+ cells is able to enhance autologous immune-mediated control of CML and that this method may be useful to develop an effective cellular immunotherapy of this leukemia.

Funding: Supported by Associazione Italiana per la Ricerca sul Cancro (AIRC), and COFIN, Ministero dell’Istruzione e Ricerca Scientifica (MIUR), Regione Campania, Intas (Brussel), EurLeukemiaNet (Brussel), Biogem (Avellino).
CO-33
FISH DIAGNOSIS AND MONITORING OF THE T(10;11)(P12;Q14)/CALM-AF10 REARRANGEMENT IN AML AND T-ALL
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CALM/AF10-t(10;11)(p12;q14) is the most frequent chromosomal change in T-cell acute lymphoblastic leukemia (T-ALL), occurring in approximately 10% of pediatric and adult cases (Asnafi et al., Blood 2003). It is also associated with acute myelogenous leukemia (AML), and with non-Hodgkin lymphoma. CD7 antigen expression and T-restricted TCRα rearrangements seem to be present also in AML, suggesting that the t(10;11)(p12;q14)/CALM-AF10 leukemias may be related to the TCRγ-δ lineage. Here we report on two pediatric cases with t(10;11)(p12;q14) and diagnosis of T-ALL and AML, respectively. Patient #1, a 13-year-old boy was referred to the Department of Pediatric Oncology because of bilateral laterocervical lymphadenopathies and gingival haemorrhage. Clinical examination revealed hepato-splenomegaly. Peripheral blood count was: Hb 11.3 gr/dL, PLT 33×10^9/L, WBC 15.5×10^9/L (with 40% of blasts). A diagnosis of ALL L1 was done on bone marrow aspirate. Immunophenotype was consistent with T-cell mature blasts positive for the following antigens: CD45, CD5, CD7, cyCD3, CD10, CD52, CD44, CD3, TdT, CD99. Patient is alive 15 months after diagnosis still on treatment (Aieop Ilo 2000). Patient #2. A 12-year-old girl was referred because of fever, headache, arthralgia, fatigue, and pallor. Clinical examination revealed splenomegaly and inguinal lymphadenopathies. Peripheral blood count was: Hb 6.1 g/dL, PLT 96×10^9/L, WBC 249×10^9/L (with 86% of blasts). Diagnosis of AML M1 was based on morphology and cytochemistry (26% of blasts myeloperoxidase positive). The following monoclonal antibodies were positive: MPO, CD45, CD7, CD44, CD11a, CD11b, CD33, CD71, CD38, and CD34. Patient underwent chemotherapy and autologous bone marrow transplantation (aBMT). Relapse occurred as bone marrow infiltration and multiple mammary nodules 11 months after aBMT. DNA clones spanning the entire CALM gene (RP11-878E11) and flanking the AF10 breakpoint (RP11-249M6 for the 3'AF10 and RP11-418C1 for the 5'AF10) were validated for FISH investigations on both metaphase cells, interphase nuclei, and cell cytospins for both diagnosis and monitoring of the malignant disorder. Funding. This work was partially supported by CNR-MIUR and FIRB.

CO-34
INSERTIONS GENERATING THE 5'RUNX1/3'CBFA2T1 GENE IN ACUTE MYELOID LEUKEMIA CASES SHOW VARIABLE BREAKPOINTS
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Translocation t(8;21)(q22;q22) is a common karyotypic abnormality detected in about 15% of Acute Myeloid Leukemia (AML) cases. The rearrangement results in fusion of the RUNX1 and CBFA2T1 genes generating a 5’RUNX1/3’CBFA2T1 transcriptionally active fusion gene on derivative chromosome 8 but some cases with ins(21;8) and ins(8;21) have been observed. However, a detailed breakpoints characterization of the insertion events has never been reported in literature. Aims. We describe 6 among 82 (7.3%) AML cases characterized by an insertion event responsible for the RUNX1/CBFA2T1 fusion. Using FISH experiments with appropriate Bacterial Artificial Chromosome (BAC) and P1 Artificial Chromosome (PAC) probes we were able to perform a detailed molecular cytogenetic characterization of 1 case with ins(8;21) and 5 with ins(21;8). Methods. The 6 cases were individuated during screening of 82 AML patients bearing the RUNX1/CBFA2T1 rearrangement, detected by RT-PCR. Bone marrow samples were studied by conventional cytogenetic analysis after 24 or 48 hours culture. Selected BAC and PAC clones were used to disclose rearrangements involving the CBFA2T1 and RUNX1 genes. In detail, the identification of the CBFA2T1 locus was...
obtained by using a mixture of BACs RP11-11808 and RP11-777J24, completely encompassing the gene; \(\text{RUNX1}\) gene was detected by PAC RP5-1107L6.

**Results.** Five cases showed a functional fusion gene on the der(21) instead of the der(8) chromosome as a consequence of ins(21;8). The insertion size was established in all cases and turned out to be very heterogeneous, ranging from a minimum of 2.4 Mb to a maximum of 44 Mb. In one case conventional cytogenetics revealed a t(8;21)(q22;q22) instead of ins(21;8)(q22;q11q22) as a large chromosome 8 region was inserted on der(21). Sequences encompassing the breakpoints on chromosome 8 were compared each other and with the region including \(\text{RUNX1}\) gene on chromosome 21, using the Genalyzer software. No significant similarity was observed.

**Conclusions.** Overall analysis of the 18 AML cases bearing ins(21;8) or ins(8;21) (12 reported in literature and the 6 analyzed in the present study) suggests that (i) this kind of rearrangement seems not to be associated with a subset of patients with common features in terms of prognosis; (ii) the insertions are not linked to the presence of additional cytogenetic rearrangements; (iii) the crucial role that \(5'\text{RUNX1}/3'\text{CBFA2T1}\) fusion gene plays in leukemogenesis does not appear to depend on breakpoint location and insertion size.

**CO-35**

**LEUKEMIA-ASSOCIATED IMMUNOPHENOTYPES SUITABLE FOR MINIMAL RESIDUAL DISEASE MONITORING ARE FREQUENT IN ADULT B-LINEAGE ALL AND PERSIST AFTER BLAST-BONE MARROW STROMA CO-CULTURE**


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We have used a multiparametric approach, based on quantitative flow-cytometry, to study whether the following leukemia-associated marker combinations are suitable for minimal residual disease (MRD) detection in adult B-lineage ALL (B-ALL). We found that positive cells for these marker combinations in normal/regenerating bone marrow (BM, 10 samples) are less than 0.01% and have peculiar physical properties that allow to discriminate normal cells from B-ALL blasts (56 BM samples). Suitable B-ALL cases, with at least one marker combination expressed by >50% of blasts, were 54/56. Among them, 32 expressed more than one marker combination. These leukemia-associated immunophenotypes were stable, as they were still detectable at the end of the co-culture of scalar dilutions of blasts with a stroma monolayer. Thus, these marker combination will be used for immunophenotypic MRD detection in adult B-ALL in the forthcoming GIMEMA therapeutic protocol.

**CO-36**

**IN VITRO TREATMENT OF AML BLAST CELLS WITH NF-κB INHIBITORS RESULTS IN THE DECREASE OF PROLIFERATION AND INDUCTION OF APOPTOSIS**


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The therapeutic results for many patients affected by acute myeloid leukemia (AML) are at present largely unsatisfactory. The overall failure of current treatments are even more disappointing in older patients who cannot be enrolled in clinical trials with conventional chemotherapy. It is therefore of great interest to identify specific molecular targets to design new therapeutic approaches. Recently, an increased NF-κB activity has been demonstrated in blast cells from AML patients. The aim of the study was to evaluate the in vitro effects of the NF-κB inhibitors in AML cell lines and in AML blast cells collected from 12 AML patients at diagnosis. Unfractioned BM cells (8 cases) or sorted blast cells (4 cases) and HL60 cell line were incubated with the proteasome inhibitors MG132 and PS341, with the IkB inhibitors Bay 11–7082 and the IKK inhibitor PS1145. The inhibition of NF-κB binding activity was evaluated using ELISA method and immunofluorescence technique with an antibody against NF-κB. The proliferation rate was evaluated by MTT assay and the percentage of apoptotic cells by flow cytometry for the detection of annexin V positive cells. In HL60 the incubations with of MG132 resulted in an inhibition of NF-κB binding activity of 40%, with PS341 of 65%, with BAY 11–7082 of 43% and with PS1145 of 23%. The MG132 was able to decrease the proliferation rate of 63%, PS341 of 69%, BAY11–7082 reduced the proliferation of 46% and PS1145 of 72%. The reduction of proliferation was associated with an increase of apoptosis of 55% with MG132, of 47% with PS341, of 56% with BAY11–7082 and 65% with PS1145. Similar results were obtained in BM MNC cells and sorted blast cells from AML patients. The incubation with MG132 or PS341 resulted in a decrease of proliferation of 50% (range 35–65%) and 55% (range 32–69%) respectively and
both increased the percentage of apoptosis to a mean value of 75%. (range 54–79%). The incubation with BAY11-7082 and PS1145 decreased the proliferation of 49% (range 37–68%) and 51% (range 39–71%) respectively. The apoptosis was increased of 57% and 65% respectively. In addition the colony growth was evaluated after incubation. The number of colonies was reduced of 89% respect to the control after incubation with MG132, of 74% with PS341, of 35% with BAY 11-7082 and 48% with PS1145. These data demonstrated that the in vitro treatment with NF-κB inhibitors is able to block the proliferation and to induce apoptosis in AML blast cells. NF-κB may therefore be considered an attractive target for a molecular therapy in AML patients not candidate for conventional chemotherapy.

**CO-37**

**DETECTION OF MLL REARRANGEMENTS INCLUDING THE MLL/AF9 FUSION IN ACUTE MYELOID LEUKAEMIA BY A SPECIFIC AND HIGHLY SENSITIVE FISH APPROACH**

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11q23 chromosome translocations involving the MLL gene occur at a 3-5% incidence in acute myelogenous leukemia (AML) and identify a sub-category of AML in the WHO classification. A number of partner chromosome were identified and the most frequent reciprocal translocation is represented by the t(9;11)(p21;q23)/MLL-AF9, accounting for 30-50% of AML with 11q23 breaks. The identification of 11q23/MLL rearrangement is important in clinical practice and it is not clear whether the t(9;11) may carry the same unfavourable prognostic significance as other 11q23/MLL breaks. Because the molecular approach to the detection of MLL rearrangements is time consuming and conventional cytogenetics may have low sensitivity, we designed a two-step fluorescence in situ hybridization (FISH) approach to the detection and characterization of MLL breaks, including the t(9;11)/MLL-AF9. Probes. We used as a first step screening PAC probes dj271a21 and dj167k13 mapping proximal and distal to the 11q23 breakpoint. These probes were labelled in green (5 prime) and red (3 prime) and carried a minimal overlap responsible for the green-yellow-red fusion signal. In those cases showing an 11q23/MLL break, a second step analysis was performed using the same MLL probes directly labelled in green and BAC probes 73e6 and 336o12 labelled in red recognising DNA sequences proximal and distal to the known AF9 breakpoints. Samples. The probes were tested on 5 normal BM controls, on 25 cases with AML without cytogenetic evidence of 11q23 translocation and on 24 AMLs with an 11q23 translocation, 8 of which had the classical t(9;11)(p21;q23). The investigator was unaware as to the result of cytogenetic analysis. Results. More than ninety-eight percent of 1000 interphase nuclei from the 5 control samples showed the expected 2 fusion signal configuration using the MLL probes, and 97.3% of 1000 nuclei showed the normal 2 green 2 red signal pattern using the MLL and AF9 probes. No case of cryptic 11q23 break was seen among 25 cases without evidence of 11q23 break at cytogenetic analysis. In one patient with sub-optimal quality of metaphase spreads an MLL break was detected by interphase FISH. An MLL split signal was observed in 56-78% of interphase nuclei in all 24 cases with an 11q23 translocation. In 1 case deletion of the telomeric portion of MLL was seen in 68% of the cells. The second step FISH experiments were performed in 10 out of 24 cases with documented MLL break, the remaining cases being currently under investigation. No evidence of MLL/AF9 fusion was detected in 6 patients with 11q23 translocation other than the t(9;11), whereas a dual fusion signal was seen in 67-91% of the cells in 4 patients with cytogenetic evidence of t(9;11). No patient with MLL/AF9 fusion showed microdeletions surrounding the MLL and AF9 genes. We arrived at the following conclusions: (i) Our first step probe system has a very high sensitivity and specificity in the detection of MLL breaks; (ii) It enables detection of MLL deletions and duplications, which may in part be missed by commercial probes; (iii) the MLL/AF9 probes detected all known cases with t(9;11), with a 100% specificity in interphase nuclei; (iv) overall, the frequency of microdeletions surrounding the MLL and AF9 breakpoints appears to be low.

**CO-38**

**INTERNAL TANDEM DUPLICATION OF BOTH MLL AND FLT3 GENES IN MYELOID LEUKEMIAS WITH TRISOMY 11**

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Introduction. Trisomy 11 as a sole chromosomal abnormality is a rare aberration observed in myelodysplastic syndrome (MDS) and/or acute myeloblastic leukemia (AML). Molecular characterization of de novo AML with trisomy 11 has shown a consistent
association with a partial tandem duplication (PTD) of the MLL gene, reported in 20% to 73% of the cases. This rearrangement leads to in-frame fusion of a portion of the proto-oncogene with itself, and this seems to represent a new genetic mechanism for leukemogenesis. Self-fusion of the MLL gene has been also observed in patients with normal karyotype, although at a much lower frequency. Internal tandem duplication (ITD) has been demonstrated as a oncogene-activating mechanism also in another gene involved in AML, namely the FLT3 gene, which encode for a receptor tyrosine kinase widely expressed in hemopoietic cells and precursors. FLT3-ITD occurs in approximately 20% of unselected de novo adult AMLs, with a higher frequency in patients with normal cytogenetics and is associated with poor prognosis in most series. Coduplication of MLL and FLT3 has been first observed by Jamal (Genes Chrom Cancer 2001) in two cases of AML, one also showing trisomy 11. Aim of the study: to evaluate the presence and the possible association of the MLL and FLT3 tandem duplication in 20 patients with myeloid malignancies carrying a trisomy 11 as a primary anomaly. Methods: Karyotype was analyzed both with standard cytogenetics and FISH with centromeric probe for chromosome11 and double-color probe for MLL gene. Southern blot was used to evaluate MLL rearrangement, and MLL-PTD was confirmed by RT-PCR (Caligiuri MA, Cancer Res 1996). FLT3-ITD was analyzed by RT-PCR (Nakao M et al., Leukemia 1996). Results: Diagnosis was AML, either de novo or secondary, in 15 patients; MDS in 4 cases; myeloproliferative disorder in accelerated phase in one case. We observed a MLL duplication in 41% of the patients with +11 (54% of AML patients). FLT3 internal tandem duplication was observed in 31% of the patients with trisomy 11 (38.5% of AML patients), with a overall incidence similar to that observed in patients with normal karyotype. However, 4 out of 5 (80%) of the FLT3-ITD cases also showed MLL-PTD, in contrast to FLT3-ITD negative cases where MLL-PTD was observed in 3/11 (27.3%). Median survival was 14.5 months for the whole group of +11 cases, 18 months for the patients negative for the MLL-PTD and only 6 months for the patients who had a MLL-PTD. Conclusions: Trisomy 11 is associated with a group of AML and MDS characterized by a poor clinical outcome; more than 40% of these patients also show the internal duplication of MLL, and these cases have an extremely poor prognosis. FLT3-ITD appears to be quite common in patients with MLL self-fusion and coexpression of the two anomalies could be explained on the basis of a common pathogenetic mechanism and/or similar response to genotoxic stress. FLT3 duplication may cooperate in determining the poor outcome observed in patients with trisomy 11.

**CO-39**

**COMBINED ANALYSIS OF BCL-2 AND MDR1 PROTEIN IN 256 CASES OF ACUTE MYELOID LEUKEMIA**


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Chemotherapy failure due to cellular drug resistance is a major problem in the treatment of acute myeloid leukemia (AML). The objectives of the study was to investigate the coordinate expression of MDR1 and bcl-2 proteins in de novo AML. The expression of the two proteins was analyzed by flow cytometry in a large series of 256 consecutive cases of AML. The results were achieved as percentage of positivity and relative mean fluorescence intensity (rMFI). To determine individual protein levels, an index which equals the product of the percentage of positive cells and rMFI, was also generated. Using a cut-off of 800 and 300 of index value for bcl-2 and MDR1, respectively, we identified 4 different classes of AML: 1) double neg; 2) single pos[bcl-2-MDR1-]; 3) single pos[bcl-2-MDR1+]; 4) double pos. The highest incidence of double neg cases was observed in the M2 class whereas double pos cases occurred more frequently in the M4, M5 and M6 subgroups. Seventy-eight percent and 71% of M0 and M1, respectively, belonged to the single pos[bcl-2+MDR1-] group (p = 0.00001). Accordingly, there was a significant association between single pos[bcl-2+MDR1-] pattern and CD34 expression (p = 0.00001). In the double pos group, 57% of the cases had a poor prognosis karyotype, 37% intermediate, and only 6% of the patients had a good prognosis karyotype (p = 0.04). Twenty-eight percent of patients belonging to the double pos category achieved CR, whereas for double neg, single pos[bcl-2+MDR1+] and single pos[bcl-2-MDR1+] category, the CR rate was 69%, 52% and 56%, respectively (p = 0.00038). In multivariate analysis, the double pos status independently affected frequency of CR (p = 0.008). In conclusions, the combined analysis of bcl-2 and MDR1 allowed different classes of AML to be identified. Bcl-2 is over-expressed in immature AML, conversely, MDR1 is over-expressed in terminally differentiated AML. However, the occurrence of the two proteins is not mutually exclusive since their coordinate expression defines a distinct subset of AML with a very poor prognosis.
CO-40
POTENTIAL USE OF CD40L+ BCP-ALL CELLS-LOADED DC TO TRIGGER ANTITUMOR IMMUNITY IN B CELL PRECURSOR ACUTE LYMPHOBLASTIC LEUKEMIA
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Childhood B-cell precursor acute lymphoblastic leukemia (BCP-ALL) is the most common form of cancer in children. Immunization strategies in cancer frequently have as common denominator the targeting of dendritic cells (DC) with its maturation stage playing a pivotal role in the development of functional CD8+ T cells and polarization of CD4+ T cells toward IFN-γ production. The finding that DC can present antigens derived from apoptotic cells prompted us to explore the feasibility of using DC pulsed with apoptotic BCP-ALL cells as adjuvants for stimulating immune responses against leukemic cells. To promote DC maturation during phagocytosis we induced the expression of CD40L on BCP-ALL cells by bystander transfer from a stable infected human bone marrow stromal cell line (HBMS) highly expressing human CD40L. After 24h of co-culture, more than 30% of BCP-ALL cells expressed CD40L. These cells were then induced to undergo apoptosis by UV irradiation, and co-cultured with immature monocytes derived-DC at 2:1 CD40L+BCP-ALL cells/DC ratio for 24 hours. At the end of incubation, DC were analyzed for CD80, CD83 and CD86 expression by flow cytometry and IL-12p70 production by ELISA. DC incubated with CD40L+ BCP-ALL cells expressed high level (more than 70%) of the maturation marker CD83, while no CD83 up-regulation was observed if CD40L- cells were used. Moreover, further maturation induced by phagocytosis of CD40L+ BCP-ALL cells resulted in higher APC activity, associated with higher expression of co-stimulatory molecules (CD80 and CD86) and higher ability to stimulate a strong proliferative response of allogenic T cells in MLR assay. In addition, CD40L+ BCP-ALL cells-loaded DC secreted IL-12p70 (0.3-2 ng/mL). In selected cases we tested the IFN-γ and IL-4 production by intra-staining analysis of naive lymphocytes co-cultured with CD40L+ BCP-ALL cells-loaded DC. In line with previous finding, these cells able to produce IL-12p70, induced a very high (30%) Th1 polarization. The capacity of CD40L+ BCP-ALL cells-loaded DC to induce anti-leukemia cytotoxicity was detected by 51Cr release assay after co-culture with T-cells in an allogenic setting. T-cells and monocyte-derived DC were obtained from healthy donors. DC were pulsed with BCP-ALL cells expressing or not CD40L. After co-culture with CD40L+ BCP-ALL cells-loaded-DC, T cells showed strong proliferation in response to leukemic cells. Moreover, T-cells cultured with CD40L+BCP-ALL cells pulsed-DC showed higher reactivity against leukemic cells than those cultured with BCP-ALL cells pulsed-DC (mean value 47.7%, range 30%-74.5% and mean value 13.5% range 0%-20%, respectively). In addition, blocking of MHC class I on target cells significantly abrogated leukemic blast lysis. In conclusion, we have demonstrated that, after phagocytosis of CD40L+ BCP-ALL cells, DC matured in potent APC able to generate cytotoxic activity against leukemic cells. Based on these preliminary findings we suggest that the DC-based vaccine may be an attractive strategy for BCP-ALL immunotherapy.
PRV-1 OVEREXPRESSION, ENDOGENOUS ERYTHROID COLONIES, AND REDUCED PLATELET MPL CONSTITUTE INDEPENDENT PHENOTYPIC VARIATIONS UNRELATED TO CLONALITY OR THROMBOSIS IN ESSENTIAL THROMBOCYTHEMIA


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Females with the monoclonal type of essential thrombocythemia (ET), based on the X-chromosome inactivation pattern (XCIP), have been shown in previous studies to present a higher incidence of thrombosis than polyclonal ones. We aimed to assess correlations between XCIP, thrombosis, and three epigenetic markers of ET, namely PRV-1 overexpression, endogenous erythroid colony (EEC) formation, and reduced platelet Mpl content. PRV-1 expression was measured by Real-Time quantitative PCR, platelet Mpl by western blotting, and EEC by standard methylcellulose cultures. Of the 100 patients enrolled, 12 were excluded because of XCIP homozygosity or the presence of an ambiguous pattern. Of the 88 subjects evaluable, fifty-three (60%) had monoclonal myelopoiesis; clinical characteristics were similar between these patients and those with polyclonal myelopoiesis. However, the frequency of thrombotic events was different in the two groups; in fact, 17 of 53 patients with monoclonal myelopoiesis (32.0%) had suffered from clinically and instrumentally documented major thrombosis, either as the initial manifestation (15 cases) or in the follow-up (2 cases), as compared to 2 out of 35 subjects (5.7%) with polyclonal myelopoiesis (p=0.009). 28/87 pts had PRV-1 overexpression, accounting for 28% and 38% of monoclonal and polyclonal pts, respectively (p=0.054). Reduced platelet Mpl was found in 71% of pts, 75% and 63% of monoclonal and polyclonal pts, respectively. There was no correlation between the presence of any of these abnormalities (nor in multivariate analysis) with XCIP or thrombotic risk. We conclude that the exploited epigenetic markers constitute independent phenotypic variations and are not clustered according to monoclonality of myelopoiesis in ET; none of them might serve as a surrogate marker of thrombotic risk in male subjects with ET as is the XCIP status in females.

GENOMIC DELETIONS ON OTHER CHROMOSOMES INVOLVED IN VARIANT T(9;22) CHRONIC MYELOID LEUKEMIA CASES


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Background. The Philadelphia chromosome (Ph), due to t(9;22)(q34;q11), is the cytogenetic hallmark of chronic myeloid leukemia (CML) and is observed in more than 95% of cases. At diagnosis, in 5–10% of CML patients the Ph chromosome is derived from rearrangements, consisting of either simple or complex variant translocations, other than the standard t(9;22). Large deletions adjacent to the translocation junction on the derivative 9 chromosome have recently been identified in patients with CML. We have documented and characterized the presence of similar deletions on the third derivative other than chromosomes 9 and 22 in 4 CML cases with variant translocations, using fluorescence in situ hybridization (FISH). Patients and Methods. We studied 11 cases affected by CML with variant translocations at diagnosis. FISH analysis was performed using P1 artificial chromosome (PAC) or bacterial artificial chromosome (BAC) probes. Cohybridization FISH experiments were performed with sets of probes designed to detect deletions flanking the breakpoint region on the third derivative chromosome. All the deletions were then studied in detail with an appropriate panel of BAC/PAC clones. A set of 16,19,22 and 12 BAC-PAC clones was used to characterize the deletions in cases #1, #2, #3 and #4, respectively. Results. Of the 11 cases, 7 (64%) bore large deletions on der(9). Among them, we found 4 cases (57%) with microdeletions also on the third derivative chromosome: t(6;9;22) (p12;q34;q11), t(9;13;22) (q34;q14;q11), t(4;9;22) (p15;q34;q11), t(9;22;11) (q34;q11;q13) in cases #1, #2, #3, and #4, respectively. In case #1 the deletion on der(6) showed a size of 2.7 Mb, with the loss of 4 known genes; among them is TNFRSF21, which encodes for a receptor that when activated, leads to the engagement of components of the apoptosis pathway. Case #2 presented a deletion on the der(13) chromosome 6 Mb in size, within which 15 known genes are located; among them, three genes are involved in the regulation of cell proliferation (RB1, RFP2, and DLEU1). Case #3
showed an extensive deletion of 20.4 Mb on der(4), with loss of 44 known genes; among them are two genes involved in cell cycle regulation: GAK (cyclin G associated kinase) and CTBP1 (COOH-terminal binding protein 1). In case #4 the breakpoint characterization on chromosome 11 allowed us to identify a 1.2 Mb deletion; 35 genes with known function are located in this deleted region. Three of these genes are involved in the apoptotic mechanism and in cancer cell proliferation: REQ, HTATIP, and CST6. Conclusions. Our study showed an association between deletions on der(9) and the loss of genomic sequences of other chromosomes involved in variant t(9;22). FISH analysis of these cases using specific PAC and BAC probes allowed us to precisely define the extension of genomic material loss and to verify which genes were deleted. The observation that in 4 cases with deletions on the third chromosome the deleted sequences included tumor suppressor gene and/or other genes involved in signal transduction or in modulation of cell proliferation, may yield further information about the pathogenesis of CML.

**CO-43**

**GENE EXPRESSION PROFILE OF CD34+ CELLS FROM IDIOPATHIC MYELOFIBROSIS PATIENTS**


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With the aim to identify differentially expressed genes and, possibly, disease-specific transcripts, CD34+ cells purified from the peripheral blood of patients with idiopathic myelofibrosis (IM) were compared to CD34+ cells purified from either the bone marrow (BM) or the G-CSF primed leukapheresis (PB) collected from normal donors. Gene expression profiling was carried out in triplicate (each sample was the pool of 5 distinct subjects) using Affymetrix HG–U133A GeneChip array, representative of 22,283 transcripts. The number of gene expressed was comparable in the two cell populations: 10,975 sequences in IM CD34+ vs 10,899 and 11,934 in normal BM- and PB-derived CD34+ cells, respectively. A number of genes were differentially expressed; of these, 343 and 151 sequences were increased in IM vs normal BM and PB CD34+ cells, respectively. On the other hand, 313 and 147 genes were decreased in IM vs normal BM and PB CD34+ cells, respectively. The prevalent biological process branches affected by these changes were traced by means of GO Mining Tool software and involved regulation of cell cycle, defense response, cell adhesion and oncogenesis. Among the genes increased in IM vs all normal CD34+ cells we found transcription factors like WT1, GAS2, (p45)N–F2, HOXA7, and ETS2; adhesion molecules/differentiation regulators, such as DLK1, vWF, TIMP3 or CD9, and receptors like leptin receptor. Among the genes down-regulated, there were IL-8, pre-B–cell colony-enhancing factor; interestingly, CXCR4, the receptor for SDF-1, was significantly reduced in IM CD34+ cells, and we speculate that this fact might be related to the high number of circulating CD34+ cells in IM. Some genes, like trophoblast glycoprotein, showed de-novo expression. These data, indicating that the expression profile of IM CD34+ cells has unique features compared to either BM or PB normal CD34+ cells, might help to identify gene(s) important for the pathogenesis of IM, and potentially useful as disease marker(s).

**CO-44**

**IMATINIB-MESYLATE AS EXPERIMENTAL THERAPY IN SYSTEMIC MASTOCYTOSIS WITH D816V C-KIT MUTATION BUT WITHOUT FIP1L1-PDGFRα FUSION TRANSCRIPT**


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Human systemic mastocytosis (SMCD) is a rare disease caused by an abnormal mast cell accumulation in various tissues. It usually occurs as a sporadic disease that is often persistent or progressive in adults, and it is often associated with eosinophilia. SM has been supposed to be associated with two classes of constitutive activating c-kit somatic mutations: the so-called enzymatic site type (EST) mutations, affecting the structure of the catalytic portion of the kinase (e. g., D816V) and the regulatory type (RT) mutations, affecting the regulation of an otherwise normal catalytic site (e. g., V560G). Recently, imatinib has proven effective in the treatment of chronic myeloproliferative disorders (CMPD) that are associated with rearrangement of the PDGFRβ with different partners, and recently in some patients with systemic mastocytosis with eosinophilia (SMCD-eos), characterised by CHIC2 gene deletion. More recently, imatinib mesylate has proven effective in the treatment of hypereosinophilic syndrome with the presence of FIP1L1–PDGFRα rearrangement. Kinase inhibitors blocking constitutive c-kit activation, such as Imatinib, might be used as therapeutic agents in SMCD, but there is increasing in vitro evidence that Imatinib is able to inhibit both wild-type and EST
mutant c-kit, but has no effect on RT mutant kit. Here, we report on six patients that met the major classification criteria for SMCD, that were symptomatic and that had the diagnosis of SMCD based on biopsy-proven and histological and immunohistochemistry evidence of Systemic Mastocytosis. We considered two patients as aggressive systemic mastocytosis (ASM), and three of them as indolent sporadic mastocytosis (ISM); one of the patients could be considered SM with an associated clonal hematologic non-mast cell lineage disease (AHNMD) classified as SM-NHL. Five of them were male and one female. Tryptase serum levels were elevated in all of them (>190 mg/L). Two patients has both absolute and perceptually elevated eosinophils in their peripheral blood (45% and 19%, respectively). xWe treated four of them with Imatinib therapy (400 mg/die) for a median period of 3 months (range 1.5-5 months). Before therapy, mastocyte cells from all of them were found positive for A816 mutant of c-Kit and negative for the presence of FIP1L1-PDGFRα, PDGFRβ-ETV6 and FGR1-BCR fusion transcripts, occasionally associated with SMCD with eosinophilia. One patient, with elevated count of eosinophils in peripheral blood, showed an initial response to Imatinib, but lost it after 1 month from the beginning of treatment. Another patient had a decrease of the mastocyte bone marrow infiltration. The remaining two patients were non-responsive. Our observations confirm the in vitro data showing that Imatinib treatment is ineffective for SM characterized by EST c-kit mutations, also if associated with hypereosinophilia.

Funding: COFIN 2003, by FIRB 2001, by the University of Bologna (60% grants), by the Italian Association for Cancer Research (A.I. R. C.), by the Italian National Research Council (C.N. R), and by grants from the Campania Region, Fondazione del Monte di Bologna e Ravenna and A. I. L.

THE ROLE OF CDK2-CDC25A-CDK2 AXIS IN THE RESISTANCE TO STI571 (IMATINIB) OF CLONAL MYELOID PROGENITORS TRANSDUCING THE P210 BCR-ABL FUSION GENE

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Unrestrained progression throughout cell cycle, mostly resulting from the abrogation of G1/S checkpoint, has a key role in the pathogenesis and progression of Chronic Myeloid Leukemia (CML). It permits, in fact, the illegitimate enlargement of clonal hematopoiesis over its normal counterpart and caus-

SODIUM VALPROATE ENHANCES IMATINIB INDUCED APOPTOSIS AND GROWTH ARREST IN BCR-ABL CELL LINES


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The molecular hallmark of CML is the expression of the chromosomal translocation t(9;22) which encodes for the tyrosine kinase Bcr-Abl. The development of the specific inhibitor of Abl tyrosine kinase activity Imatinib (formerly STI-571, Novartis, Basel, Switzerland) completely revolutionises the therapy and the prognosis of CML patients. Recent clinical trials have shown that Imatinib induces complete hematological responses in 95% of chronic phase CML patients. Unfortunately, complete cytogenetic responses are not achieved in about 30% of Imatinib-treated CML patients. Moreover in accelerated phase and in blast phases, Imatinib appears to be less effective. The persistence of neoplastic cells expressing t(9;22) translocation during Imatinib treatment suggests that sooner or later a clonal expansion could occur with a relapse of the disease. So additional therapies are required to completely eradicate residual Bcr-Abl positive cells during Imatinib therapy. HDAC inhibitors are a new and promising class of anticancer drugs, which show impressive activities in many cell types. Recent evidences have attributed to HDAC inhibitors the role of enhancers activities in many cell types.

The aim of the present study was to evaluate the ability of Valproate to increase Imatinib-induced apoptosis and growth arrest in CML cell lines and in CML patient’s bone marrow samples. In particular we have treated the Bcr-Abl positive Imatinib-sensible and resistant K562, KCL-22 and CML-T1 with 5 microM Valproate, 0.5 μM Imatinib and with the combination of the two drugs. After 48 hours of incubation, cell growth (cell count) and apoptosis (quantified by Cell Death Detection Elisa) have been evaluated. The association Imatinib plus Valproate enhances Imatinib induced growth arrest and apoptosis in Imatinib sensible cell lines. In Imatinib resistant K562 and KCL-22 cell lines, the exposure to 0.5 microM Imatinib does not affect proliferation nor apoptosis, but when associated with Valproate, basal proliferation is reduced of about 50% with a sustained induction of apoptosis. An important effect of Imatinib is the abrogation of CML multilineage colony-forming units (CFU-Mix), granulocyte macrophage-colony-forming unit (GM-CFU) and erythroid burst-forming unit (BFU-e) colony formation. In Imatinib resistant patients, Imatinib does not affect colony formation of bone marrow samples. We have isolated bone marrow mononuclear cell from two informed patients, in hematological and cytogenetic resistance to Imatinib, to test the clonogenic potential in the presence of Valproate, Imatinib and the association of the two drugs. Imatinib alone have inhibited minimally the growth of CFU-Mix, GM-CFU and BFU-e colonies but when Imatinib is combined with Valproate the number of colonies is sensibly reduced. Bone marrow mononuclear cells of the same patients have been treated for 24 hours with Valproate, Imatinib and the association of the two drugs. At the end of the incubation, apoptosis has been evaluated by flow cytometry detection of Annexin V. The exposure to Imatinib and Valproate alone do not affect apoptosis of the cells while the combined exposure determines an increase of annexin V positive cells, suggesting an important induction of apoptosis. Western blot analysis shows that the association of the two drugs does not directly interfere with the phosphorylation state of Bcr-Abl and its signal transduction. In conclusion, these data suggest that the combined therapy, low dose Imatinib plus Valproate, could enhance the Imatinib-induced apoptosis and growth arrest. This strategy could contribute to a complete eradication of residual Bcr-Abl positive clones in those patients in which complete cytogenetic remission is not reached during Imatinib treatment.

**CO-47**

**THE IN VITRO TREATMENT WITH NF-kB INHIBITORS IS ABLE TO OVERCOME IMATINIB RESISTANCE IN CELL LINES AND CML RESISTANT PATIENTS**

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Several data demonstrated that Imatinib resistance may be due in same patients to the presence of BCR-ABL independent signals responsible for the survival of the Ph-positive clone despite effective targeting of BCR-ABL. It is therefore of great relevance to identify alternative molecular targets to block the oncogenic pathway in these subset of patients. An increased NF-kB activity has been demonstrated in CML cells and in K562 cells. The aim of the study was to evaluate the effects of the NF-kB inhibitors in cell lines and in CML patients sensitive and resistant to Imatinib therapy both alone or in combination with this latter. We incubated K562 and KCL both sensitive (s) and resistant (r) cells and the BM cells collected from 6 cytogenetic resistant patients, two of them also hematological resistant with the proteasome inhibitors MG132, PS341 and the IKK inhibitor Bay 11-7082 and IKK inhibitor PS1145. The inhibition of NF-kB binding activity was evaluated using ELISA method and immunofluorescence technique with an antibody against NF-kB. The proliferation rate was evaluated by MTT assay and the per-
percentage of apoptotic cells by flow cytometry for the detection of annexin V positive cells. In K562s Imatinib incubation reduced the proliferation rate of 48%, MG132 of 45%, BAY11-7082 of 40% and PS1145 of 65%. The reduction of proliferation was associated with an increase of apoptosis, 30% with Imatinib, 25% with both proteasome inhibitors, 28% with BAY11-7082. Similar results were obtained in KClr cells. By contrast Imatinib incubation of K562r and KClr did not result in a significant inhibition of proliferation. By contrast, the proteasome inhibitors induced a reduction of proliferation of 70%, BAY11-7082 and PS1145 of 10% and 22% respectively. Interestingly, the combination of Imatinib with MG132 or PS341 induced a reduction of proliferation of 82% and 89% respectively, and the induction of apoptosis 54% and 40%. The association of Imatinib with BAY11-7082 resulted in a reduction of proliferation of 87% and an increased of apoptosis of 59% and the association with PS1145 reduced the proliferation of 87% and increased apoptosis of 67%. Similar results were obtained in CML patients resistant to Imatinib. The association of Imatinib with MG132 resulted in a decrease of proliferation of 47%, (range 35–64%) and increase of apoptosis of 62%. (range 51–89%). Similar results were obtained with the association of PS341 (mean reduction of proliferation of 49%). The association with BAY and PS1145 decreased the proliferation of 57 and 61% respectively (range 35–69% for BAY and 29–72% for PS1145) and both increased apoptosis with mean value of 56% and 60%. Moreover the colony growth obtained from BM cells after incubations was inhibited of 70% and 81% with the association of Imatinib with MG132 and PS341, and was inhibited of 61 and 79% with the association with BAY and PS1145 respectively. Finally, these data were confirmed by the experiments performed on K562 cells transfected with Ikb super-repressor which blocks NF-kB in the inactive status. In line with the results described, these cells showed a proliferative arrest and an increase of the apoptosis rate. We conclude that NF-kB inhibitors present a potential activity in Imatinib resistant cells and particularly, the combination of Imatinib and NF-kB inhibitors is able to strongly block the proliferation and to induce apoptosis in cell lines and CML patients resistant to Imatinib therapy. The combination of Imatinib and NF-KB inhibitors may therefore represent an attractive therapy for CML resistant patients.

**CO-48**

**PREDICTION OF RESPONSE TO IMATINIB BY PROSPECTIVE QUANTITATION OF BCR-ABL TRANSCRIPT IN LATE CHRONIC PHASE CHRONIC MYELOID LEUKEMIA PATIENTS**


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Imatinib mesylate (STI571), a specific Bcr-Abl tyrosine-kinase signal-transduction inhibitor, has shown antileukemic activity in clinical studies, becoming the standard therapy for Philadelphia chromosome-positive (Ph+) chronic myeloid leukemia. Prediction of response to imatinib cannot be anticipated with certainty by repeated examination of bone marrow metaphases for the presence of the Ph chromosome. The quantitative reverse-transcriptase polymerase chain reaction (QRT-PCR) has proved extremely valuable for assessing and monitoring minimal residual disease in patients who achieve Ph negativity with imatinib mesylate, but few data are available on the use of QRT-PCR for predicting response to the drug. We used this method on 191 out of 324 chronic myeloid leukemia patients in the late chronic phase entered into a phase II clinical trial conducted by the Italian Cooperative Study Group on Chronic Myeloid Leukemia with imatinib 400 mg/day administered orally as second-line therapy. Bone marrow samples were collected before treatment, after 3, 6 and 12 months or at the end of study treatment (12 months) while peripheral blood samples were obtained after 2, 3, 6, 10, 14, 20 and 52 weeks of therapy. QRT-PCR analysis was standardized and performed in three different laboratories, and the amount of Bcr-Abl transcript was expressed as the ratio of Bcr-Abl to β2-microglobulin (β2M). Before imatinib therapy, we found a different amount of neoplastic transcript in bone marrow and peripheral blood (p<0.001), reflecting the higher percentage of myeloid precursor in bone marrow. In the nonresponders, the Bcr-Abl:β2M ratio in bone marrow remained substantially during the treatment, while in patients obtaining a complete, stable cytogenetic response...
the transcript ratio decreased significantly from 0.246 baseline to 0.0062 after 3 months, to 0.0009 after 6 months and to 0.0008 after 12 months. Analysis of blood samples was as informative as bone marrow at 3, 6, 9 and 12 months. At 2 months (+57 days) after the start of imatinib therapy the differences between no cytogenetic response and a complete, stable cytogenetic response were detectable, thus affording an early prediction of the karyotypic response to imatinib. We show that, following initiation of imatinib, the early Bcr-Abl level trends in both bone marrow and peripheral blood samples made it possible to predict the subsequent cytogenetic outcome after 6 and 12 months of treatment, and that these early trends were also predictive of progression-free survival.

Funding: Supported by: COFIN 2003, by FIRB 2001, by the University of Bologna (60% grants), by the Italian Association for Cancer Research (A. I. R. C.), by the Italian National Research Council (C. N. R), and by grants from the Campania Region, Fondazione del Monte di Bologna e Ravenna and A. I. L.

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CO-49

IMMUNOGLOBULIN MUTATIONAL STATUS IN PARAIMMUNOBLASTIC EVOLUTION OF CHRONIC LYMPHOCYTIC LEUKEMIA


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The paraimmunoblastic variant is a rare subtype of B-cell small lymphocytic lymphoma/leukemia (SLL/L), accounting for approximately 1–2% of cases of B-SLL/L. It is characterized by the diffuse proliferation of cells normally populating the pseudo-proliferating center, the so-called paraimmunoblasts named by Lennert. These are mitotically active cells and have a distinct cytological appearance, characterized by an high nuclear/cytoplasmic rate, vesicular nuclei and central prominent nucleoli. The clinical course of the disease is aggressive, refractory to the standard chemotherapy and rapidly fatal. Data from the literature well defined the morphological appearance, but a complete immunophenotypic and molecular characterization of this lymphoproliferation is lacking. Here we report 7 cases of a B-cell lymphoproliferative disorder with morphologic and clinical features consistent with the paraimmunoblastic variant of SLL/L. All cases presented with hepatosplenomegaly, lymph node enlargement or peripheral lymphocytosis. Immunophenotypic analysis showed that the lymphoid cells expressed the CD5, CD19 and FMC7 but not the CD23 antigens. The morphologic appearance and positivity for CD5 and FMC7 help to distinguish this entity from prolymphocytic evolution of chronic lymphocytic leukemia and from mantle cell lymphoma, respectively. The mutational status of immunoglobulin genes was defined analysing the nucleotide sequence of rearranged heavy chain variable region (IgVH) obtained by direct sequencing of RT-PCR products in all 7 cases. Considering the classical VH homology cut off value of 98%, 4 patients had unmutated and 3 had mutated VH genes. All mutated cases had a germ line homology rate between 96% to 98%. Three cases had VH1 family, in particular VH1-69, VH1-3 and VH1-2, two cases had VH3 family, VH3-15 and VH3-21 in a mutated and an unmutated patients respectively, and two cases had VH4 fami-
ly, VH4-61 and VH4-39. The majority of patients studied used D6 segments (5 of 7), in particular D6-13 was found in three cases. CDR3 length ranged from 11 to 21 amino acids. The sequence of VH genes was determined both at diagnosis and after paraimmunoblastic evolution, no differences in mutational pattern have been identified. Furthermore, molecular analysis of BCL2 and BCL1 genes is also ongoing to investigate whether additional genetic lesions may account for the biologic and clinical aggressiveness of this disease. These date suggest that paraimmunoblastic evolution can arise in both mutated and unmutated cases. Nevertheless, VH status was characteristic of CLL-patients with worse clinical outcome of the disease. No evolution in the IgH rearrangements was evident in both VDJ usage and somatic mutations, suggesting no evident role of germinal centre ambient in this rare type of CLL progression.

**CO-50**  
**RELATION BETWEEN GENE EXPRESSION PATTERN, IMMUNOGLOBULIN MUTATIONAL STATUS AND CLINICAL OUTCOME IN CHRONIC LYMPHOCYTIC LEUKAEMIA**  
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B-cell chronic lymphocytic leukaemia (B-CLL) is a lymphoproliferative disorder characterized by transformed monoclonal B-cells with the appearance of small mature lymphocytes and with a characteristic immunophenotype, typically positive for CD5, CD23 and CD19 and negative for surface CD22 and FMC7 with low expression of IgM/IgD surface immunoglobulins. The relentless accumulation of this small mature B-cells, which have escaped programmed cell death and have undergone cell cycle arrest in the G0 phase, is the hallmark of B-CLL. Although the median survival of patients with this form of leukaemia is around 10 years, in individual patients the prognosis is extremely heterogeneous, ranging from a very short to a normal lifespan. Despite having several phenotypic characteristics of naïve B1a cells, CLL have been shown to have somatically mutated immunoglobulin variable region genes in more than 50% of cases. According to this, CLL patients can be divided into two subgroups with different clinical behaviour and survival. Here, we report the expression levels analysis of specific genes known to be differentially expressed in CLL subsets as well as of different genes involved in lymphocytes differentiation and in the germinal centre reaction according to the immunoglobulin VH mutational status. The status of Ig genes was assessed analyzing the nucleotide sequence of rearranged heavy chain variable region (IgVH) obtained by direct sequencing of RT-PCR products of 61 well characterized B-CLL cases. Considering the classical VH homology cut off value of 98%, 31 (51%) had mutated (M-CLL) and 30 (49%) had unmutated (UM-CLL) VH genes. The similarity of the VH genes to the closest germ line genes ranged from 83% to 100%. Inside mutated group, 6 patients (9.8%) had a germ line homology rate between 95% to 98% and that group was indicated as low mutated (LM-CLL). In common with several other series, we evidenced in the unmutated group an overuse of VH1 family that was found in 13 patients (21.3% of total cases and 43.3% of unmutated one). The VH1-69 was exclusively used in unmutated CLL (n=8) and demonstrated the highest frequency accounting for 26.7% of unmutated VH genes. On the contrary, there was a significant tendency for VH3 and VH4 family genes (p=0.001) to be used by the subset that have mutations. In particular, mutated CLL evidenced an elevated frequency of VH3-23 and VH4-34 family accounting together for 38.8% of cases. VH3-21 was only used in two occasions in unmutated CLL. Again, we found a significant difference in CDR3 length that was longer in unmutated VH genes (mean=17.6 aa) than in mutated cases (mean=12.7 aa) (p=0.0001). UM-CLL patients had a significant tendency to present at diagnosis at an higher Binet stage. Then, most stage A patients (78.6%) expressed mutated VH genes, instead most stage B and C patients (76.5% and 100% respectively) had unmutated VH genes (p<0.0001). Mutational status well correlated with progression-free survival showing a much more frequency of rapid disease evolution in patients with unmutated VH genes. Moreover, UM-CLL patients seem to have a more aggressive disease requiring treatment in the great majority of cases (67.9%) in contrast with only 30% of M-CLL patients (p=0.012). Large scale gene expression analysis was assessed by microarray technology in order to identify specific gene expression profile related to mutated or unmutated immunoglobulin status. Total RNA from 30 cases (14 M-CLL and 16 UM-CLL) was converted into Cy5-labelled cRNA and co-hybridized with a Cy3-labelled reference (mix of 7 M-CLL RNA and 7 UM-CLL RNA) to Agilent Human 1A Oligo Microarray representative of about 18’000 well characterized genes. First, all gene expression data were compared each other using unsupervised hierarchical clustering analysis in order to identify genes belong-
ing to apoptotic, proliferation, BCR signal transduction and other signatures that could be involved in determining transformed cell phenotype. Then, gene expression data were compared each other using support vector machine to identify genes most discriminating M-CLL and UM-CLL groups in order to create a gene-based predictor. Finally, expression of some genes involved in BCR signal transduction and germainal centre reaction were quantified by Real Time PCR. A significant statistical correlation between UM-CLL cases and high expression of BCL6, CD38 and ZAP-70 genes was assessed. The same B-CLL cases were also studied by flow-cytometry for CD38 protein levels expressed both as the percentage of CLL positive cells and as antibody-binding capacity (ABC). Taken together CD38 data were able to divide B-CLL patients in groups with peculiar level of expression and clinical outcome. Again, RNA and protein level of genes involved in cell cycle regulation as p27, CDK3 and Cul-1 were analysed and compared each other and with mutational VH status. Data analysis revealed that the more statistically relevant parameters related to VH status were CD38 and ZAP-70 mRNA expression levels as determined by RealTime PCR.

**CO-51**

**THE ZAP-70 TRANSCRIPT AMOUNT EVALUATED BY RQ-PCR IN PERIPHERAL BLOOD OF CLL PATIENTS CORRELATES WITH THE IgVH MUTATIONAL STATUS**

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The mutational status of immunoglobulin heavy-chain variable-region (IgVH) genes in the leukemic cells of chronic lymphocytic leukemia (CLL) is an important prognostic factor in the disease. We investigated whether the quantitative assessment of ZAP-70 transcript amount in CLL cells correlated with the IgVH mutational status. The expression level of ZAP-70 was analyzed in peripheral blood (PB) mononuclear cells separated on a ficoll- hypaque density gradient, obtained from 42 CLL. Twenty-two out of 42 patients showed the mutation of IgVH and 20 were unmutated. The expression level of ZAP-70 was established using quantitative RealTime PCR using a specific set of primers and probe (Assays-on-Demand, gene expression products, Applied Biosystems). The values obtained were normalized using ABL as housekeeping gene and the final results were expressed using the Delta /Delta method. The final numerical values are expressed as 2(e)-Delta/DeltaCt. We stratified the patients according to the presence or absence of IgVH mutations and we analyzed the ZAP-70 transcript amount in the two groups. The unmutated IgVH samples showed significantly higher levels respect to the unmutated (p=0,0003 by t test) with a mean value of 2_deltaCt =0,545 (range 0,22-1). The mean value of mutated samples was 0,1875 (range 0,04-0,33). These data allow to establish that all the patients who present ZAP70 values above the value of 2_deltaCt of 0,40 belong to the group without the presence of IgVH mutation while all the patients who present a ZAP70 value of 2_deltaCt below 0,2 belong to the group with IgVH mutation. A small number of patients, 6 out of 42, (14%) show values between 0,2 and 0,4, 3 of them present the IgVH mutation and 3 are unmutated. These data allow to establish that the quantitative assessment of ZAP-70 performed on unfractioned MNC cells from peripheral blood of CLL patients correlate with the presence of IgVH mutations and it may therefore be considered a rapid and easy surrogate of the mutational analysis in the vast majority of patients. For the small fraction of borderline patients the analysis of IgVH mutations may probably represent the best choice, limiting in this way the number of candidate patients for this analysis.

**CO-52**

**TELOMERE LENGTH PREDICTS TIME TO FIRST TREATMENT AND TIME TO PROGRESSION IN B-CELL CHRONIC LYMPHOCYTIC LEUKEMIA**

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Telomeres are repeated DNA sequences at the end of chromosomes. In a recent study we demonstrated that telomere length (TL) strongly correlates with the histopathogenesis of lymphoproliferative disorders and in particular to their origin in relation to germinai center (GC) (Ladetto M et al., Blood 2004 e-pub). The main prognostic feature in B-cell chronic lymphocytic leukemia (B-CLL) is V-IgH mutation status which discriminate V-IgH-mutated GC-experienced CLL (good prognosis) from V-IgH-unmu-
tated GC-unexperienced CLL (poor prognosis). We thus decided to evaluate the prognostic role of TL in B-CLL and correlate it with V-IgH mutational status. Aims of this study were: i) to assess the prognostic significance of TL in B-CLL patients; ii) to verify whether there is a relationship between TL and V-IgH mutational status in B-CLL patients. We analyzed telomere restriction fragments (TRF) and V-IgH rearrangements in 83 untreated B-CLL patients. Fifty-four were male and 29 female, their median age was 54 years (range 38–87). Forty-seven patient were in stage A of Binet, 24 patients were in stage B and 12 patients had a poor clinical prognosis (stage C of Binet). TL was evaluated by Southern blot and mutational status of V-IgH rearrangement by direct sequencing. Both approaches have been previously described (Ladetto M et al, Blood 2004 e-pub). Sequences with <or=2% deviation from any germ line V-IgH sequence were considered GC-unexperienced while sequences containing >2% deviation from any known germ line Ig-VH were considered GC-experienced. Survival analyses were performed using the Kaplan–Meier method. Overall, median TRF was 5775bp (range 1737–9844bp). There were no correlation between TL and patient age, sex and clinical presentation. A cut-off of 4500bp discriminated two subgroups of patients characterized by different clinical outcome both in terms of time to first treatment (TFT) and time to progression (TTP). Patients with TL <4500bp had a median TFT of 16 months and a median TTP of 14 months while patients with TL >4500bp had a median TFT of 36 months and a median TTP of 36 months (p<0.05 and p<0.01, respectively). V-IgH sequencing is currently available in 54 patients. The previously defined cut-off of 4500bp effectively segregated patients according to V-IgH mutational status. 100% of mutated patients had TRF length >4500bp while 63% of unmutated patients had TRF length <4500bp. Of note, the 9 discordant patients (unmutated V-IgH gene with TRF >4500bp) had a clinical outcome comparable to patients with a V-IgH mutated status (median TFT of 30 months and median TTP of 80 months). Our data demonstrate that: 1) TRF length has prognostic significance in B-CLL; 2) TL in B-CLL correlates with V-IgH mutational status; 3) it is possible that when discordance exists between these two biological parameters, the clinical behavior could be better predicted by TRF length compared to V-IgH mutational status.

CO-53

QUANTITATIVE PCR OF BONE MARROW BCL2/IgH POSITIVE CELLS AT DIAGNOSIS PREDICTS TREATMENT RESPONSE AND LONG-TERM OUTCOME IN FOLLICULAR NON HODGKIN’S LYMPHOMA

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By real time quantitative PCR reaction (RQ-PCR) we evaluated BCL2/IgH+ cells in the bone marrow (BM) and peripheral blood (PB) of 86 Follicular Lymphoma patients treated with the sequential administration of CHOP and Rituximab. At diagnosis, the amount of BCL2/IgH+ cells in the BM was low (1 BCL2/IgH+ cell in 103 - 105 normal cells) in 43% of patients, intermediate (1 in 102 normal cells) in 34% or high (>1 in 102 normal cells) in 23%. A 2 log decrease of BCL2/IgH+ cells was achieved after CHOP and additional 2 logs following Rituximab. By multivariate analysis, a low level of BCL2/IgH+ cells in the BM at diagnosis, was the best predictor for the achievement of a complete clinical and molecular response. At 5 years, the Event Free Survival of patients with a low or high tumor infiltration in the BM is 59% and 32% respectively. The Freedom From Recurrence of patients who achieved a molecular response in the BM, no matter whether after CHOP alone or CHOP and Rituximab, is 65% as compared to 33% of patients who did not (p<0.006). RQ-PCR performed on BM samples predicts treatment response and long-term clinical outcome in follicular lymphoma patients.
The anti-CD52 antibody alemtuzumab has been approved for the treatment of fludarabine refractive B-CLL. We have investigated its mechanism of action in vitro against B-CLL and B-NHL, and compared it to rituximab. Standard human complement cytotoxicity assays (CDC) were performed on freshly isolated neoplastic cells. Alemtuzumab lysed the 23 B-CLL samples through complement activation (mean 80%) much more efficiently than rituximab (mean 16%), presumably due to the higher expression levels of CD52 compared to CD20 (mean MFI of 1450 compared to 185, respectively). All other leukaemic B cells, including 1 PLL, 2 HCL and 6 B-NHL were effective targets for both antibodies, with 88% and 85% mean lysis, respectively. Both CD52 and CD20 were highly expressed in these cells (MFI>500 for CD52 and >400 for CD20). ADCC was also studied, using standard 51Cr release assays, using peripheral blood mononuclear cells as effector cells, before and after 2-days culture with 1000U/ml IL-2. In contrast to CDC, B-CLL and most B-NHL samples were poorly lysed through ADCC using freshly isolated PBMC as effector cells, with either monoclonal antibody and regardless of target antigen levels. Antibody dependent lysis could however be significantly increased by culture of the effector cells with IL-2. We conclude that CDC is likely to be an important mechanism of action of alemtuzumab in B-CLL and that combination with IL-2 may increase its efficacy through ADCC. Other mature neoplastic B cells are good targets for alemtuzumab and complement mediated lysis, making the antibody a good candidate to treat minimal residual disease in the context of non-myeloablative BMT in B-NHL and B-CLL.

We studied a scheme of PBSC mobilization with Rituximab and high-dose cytarabine in 36 patients with relapsed/refractory follicular lymphoma (25 grade 1, 7 grade 2, 3 grade 3, 1 transformed) and 7 with refractory mantle cell lymphoma enrolled in a program of high-dose chemotherapy and autotransplant. 13 patients were females and 30 males. Median age of the whole series was 50 years (range 34-64). After two courses of debulking immuno-chemotherapy with rituximab, vincristine and cyclophosphamide (R-CV), we used a combination of rituximab 375 mg/m² on day 1 and cytarabine 2 g/m² every 12 hours on days 2 and 3. A second infusion of rituximab 375 mg/m² was given on day 9. Granulocyte colony stimulating factor (G-CSF) 5 mcg/kg/day subcutaneously was started on day 6. No adverse events occurred during Rituximab administration. No grade III-IV non-hematologic toxicities were observed after HD-AraC; three episodes of grade 3-4 granulocytopenia were registered. Patients needed a median of 1 platelet transfusion (range 0-4) and a median of 0 erythrocyte transfusion (range 0-3). Patients were hospitalised for 3 days (for the first Rituximab infusion and the two days of HD-AraC). All the other procedures, including leukaphereses, were performed on an outpatient basis. Leukaphereses were started after a median of 13 days (range 11-16) after the first dose of HD-AraC. The median number of CD34+ cells collected was 22.48×10⁶/kg (range 4-73.2). Of 23 patients molecularly informative at start of salvage treatment (PCR-positive for bcl-2 or bcl-1 in blood and marrow), all collected PCR-negative cells. Monitoring of peripheral CD19+ and CD20+ B-cells prior to, and throughout the purging period showed that a preparative phase with Rituximab, Vincristine and Cyclophosphamide determines a profound depletion of B-cells in peripheral blood. B-cell depletion persists during the mobilisation phase with Rituximab and high-dose cytarabine allowing a collection of PBSC free of B-cells (median CD19+ and CD20+ cells counts 0%). In conclusion, in patients with indolent lymphoma, the concurrent administration of Rituximab and high-dose cytarabine is an efficient method for obtaining in vivo purged lymphoma-free PBSC.

**CO-55**

**B-CELL DEPLETION AND MOBILIZATION OF LYMPHOMA-FREE PBSC WITH RITUXIMAB AND HIGH-DOSE ARA-C IN FOLLICULAR AND MANTLE CELL LYMPHOMA**

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**CO-56**

**MICRODISSECTION OF LYMPHOMA CELLS IN HCV-ASSOCIATED PRIMARY SPLENIC LYMPHOMAS INDICATES A DIRECT PATHOGENETIC ROLE OF VIRAL INFECTION**

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Epidemiological evidences indicate that Hepatitis C virus (HCV), besides liver diseases, is associated with extrahepatic diseases, including B cell lymphoproliferative disorders, such as type II mixed cryoglobulinemia (usually a benign disorder), and overt B cell non-Hodgkin’s Lymphoma (NHL). A recent report on regression of splenic marginal zone lymphoma after anti-viral treatment with interferon and ribavirin has significantly strengthened the cause-effect relationship between HCV infection and lymphoma. The molecular basis for viral induced B cell proliferation is still unknown. One possibility is that HCV stimulates the proliferation of monoclonal B cells via their HCV-specific B cell receptor (BCR) on the cell surface and binding of the HCV envelope proteins (E2) to a cellular ligand, CD81, may also enhance this antigen-driven process. To investigate the relationship between HCV infection and NHL, we used Laser Capture Microdissection (LCM) to isolate the morphologically normal and neoplastic cells in spleen paraffin-embedded sections of 12 patients with primitive splenic B-NHL. Eight out of 12 patients evaluated in this study, had high-grade (HG) NHL, and the remaining 4 had low-grade (LG) NHL. Serum anti-HCV antibodies were detected in 5 out of the 8 HG NHL patients and in 3 out of the 4 LG NHL patients. LCM is a recently developed technique that couples laser-assisted dissection of cell from tissue sections with microscopic visualization of the samples and allows rapid and reliable isolation of pure cells populations, in one step, under direct microscopic visualization. Using LCM, the normal and malignant germinal centre cells were isolated from the same patient for subsequent analysis. We firstly search for the presence and distribution of HCV and found that in HCV positive LG NHL patients, HCV-core mRNA was detectable by RT-PCR nested analysis only in morphologically neoplastic germinal centre cells of the microdissected spleen samples, but not in morphologically normal spleen cells of the same patients. On the other hand, cells dissected from both normal and neoplastic areas of spleen tissue section from HCV positive HG NHL patients showed the presence of HCV-core mRNA at RT-PCR nested analysis. The high tendency of HG LNH cells to circulate among the focal sites at spleen and both the blood and lymphatic circulation systems may explain this latter result, being the apparently normal areas of the spleen tissue section infiltrated with lymphoma cells. In HCV positive LG NHL patients with assessed the expression of the BCL2 mRNA that encodes for an antiapoptotic protein up-regulated in CLL and LG NHL patients. Interestingly, we found that as expected, the case of primary splenic HCV negative LG NHL, the levels of expression of this gene was rather high, while in all the HCV positive patients, neoplastic germinal centre cells had very low levels of this mRNA. The expression of BCL2 mRNA was uniformly low in both HCV positive and HCV negative HG NHL spleen tissue sections. Taken together, these results support the direct role of HCV infection in the lymphoma transformation in the case of primary splenic LG NHL, and indicate that the mechanisms of transformation in HCV infected cells may be different respect the HCV negative cases, while at the moment it is less clear the role HCV infection in the HG grade NHL.

Funding: Supported by Associazione Italiana per la Ricerca sul Cancro (AIRC), and COFIN, Ministero dell'Istruzione e Ricerca Scientifica (MIUR), Regione Campania, Intas (Brussel), EurLeukemiaNet (Brussel), Biogem (Avellino).
**CO-57**

**CYCLOOXYGENASE-2 (COX-2) EXPRESSION IS COMMON IN MULTIPLE MYELOMA AND ACTS AS AN ADVERSE PROGNOSTIC INDICATOR**

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**Introduction:** A perturbed microenvironment with secretion of inflammatory cytokines is a typical feature of multiple myeloma (MM). Prostaglandins are among the most important mediators in inflammation and angiogenesis and play a major role in supporting tumor growth in several solid malignancies. Expression of cyclooxygenase-2 (COX-2), the key enzyme of prostaglandin synthesis in inflamed tissues, has been documented in many of these cancers and plays a major role in their development. In addition, COX-2 expression often acts as a prognostic indicator associated with poor outcome. Despite a huge amount of data concerning COX-2 expression in solid tumors, few data are currently available on its role in hematological malignancies. Particularly in MM there are several biological, epidemiological and clinical considerations suggesting a potential involvement of the prostaglandin pathway. Aim of this study is to verify the potential involvement of COX-2 in MM and to assess the potential prognostic role of its expression. Patients and methods: COX-2 expression has been assessed by western blotting as previously described (Du Bois RN, et al, Gastroenterology, 1996, 110:1259-1262). Our positive control was the COX-2 positive cell line HT-29, while peripheral blood and bone marrow (BM) from 15 healthy donors were employed as negative controls. We assessed a panel of 115 samples obtained by 97 patients and methods: COX-2 expression was not observed in the 15 normal BM. In contrast, COX-2 expression was noticed in 14% of MGUS, 35% of MM at diagnosis, 20% of MM in remission and 46% of MM at relapse. COX-2 positivity at diagnosis and relapse appeared to be unrelated to disease stage, BM plasmocytosis, creatinine, Hb levels, β2 microglobulin. COX-2 expression appeared to be prognostically important: among patients at diagnosis the median time to progression was 17 months in COX-2 positive and 32 months in COX-2 negative subjects (p<0.000). Among 11 patients showing COX-2 expression at relapse 7 have already died, while four are still alive (median observation time: 6 months, range 1-31). Of 14 COX-2 negative patients only two patients died while 12 are still alive (median observation time: 8 months, range 1.5-99). Immune-histochemistry and cell separation studies indicate that COX-2 expression is mostly related to the plasma cell population. Conclusions: a) COX-2 is frequently expressed in plasma cell dyscrasias; b) COX-2 expression is more frequent in advanced disease phases; c) COX-2 expression seems related to a worse outcome. Future studies are required to verify whether COX-2 might be useful for clinical purposes as a prognostic marker and/or therapeutic target.

**CO-58**

**A VEGF-DEPENDENT AUTOCRINE LOOP ON ENDOTHELIAL CELLS PROMOTES ANGIOGENESIS AND PROGRESSION IN MULTIPLE MYELOMA**


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Angiogenesis is an important component of bone marrow stroma in multiple myeloma (MM). It parallels the transition from monoclonal gammopathy unassociated/unattributable (MG[u]) to MM, or from remission MM to relapse and the leukemic phase. In addition, the neovessels convey oxygen and metabolites, while endothelial cells (EC) at their tips secrete growth and invasive factors for plasma cells. The mechanisms that induce the formation and sprout-
The bone marrow by magnetic microbeads coated with Ulex europaeus-1 (UEA-1) lectin, and cultured in RPMI-1640 medium supplemented with 10% FCS and 1% glutamine. Cell were studied for their phenotype and cytokine production by western blot, RT-PCR and ELISA. EC were also studied for the activation of transduction pathway linked to VEGFR-2 by immunoprecipitation and western blot. Functional studies were also done: EC proliferation in short-term cultures, Matrigel capillary assay, evaluation of MMP-2, MMP-9 and uPA activities. Inhibition experiments were done by incubation with neutralizing anti-VEGF and anti-VEGFR-2 antibodies. Higher expression of VEGFR-2 and VEGF in MMEC than MG(u)EC and HUVEC was demonstrated by RT-PCR, western blot and ELISA. Western blot of extracts from EC cultured in starvation medium showed that MMEC, but not (or marginally) MG(u)EC and HUVEC, display constitutive autophosphorylation of VEGFR-2 and the ERK-2 associated kinase. When the medium was added with neutralizing anti-VEGF-A and especially anti-VEGFR-2 MoAbs, phosphorylation was reduced and the two inhibitors had an additive effect. As expected, addition of VEGF-A enhanced the phosphorylation and this, too, was reduced by the inhibitors in a similar fashion. MMEC displayed higher proliferative activity than MG(u)EC or HUVEC and it was reduced significantly by anti-VEGF-A and, more strongly, by anti-VEGFR-2 MoAbs. MG(u)EC and HUVEC proliferation was not substantially modified by these inhibitors. After a 12-hour incubation, MMEC had spread through the Matrigel surface and aligned to form branching, anastomosing tubes with MMEC, but not (or marginally) MG(u)EC and HUVEC, displaying co-expression at higher levels of VEGF and VEGFR-2 in MMEC vs MG(u)EC and HUVEC. VEGF-dependent autocrine loop is operative in MMEC. VEGFR-2 inhibitors may thus prove effective in the management of MM by targeting both plasma cells and newly-formed vessels.

Multiple myeloma (MM) is a malignant proliferation of bone marrow plasma cells characterized by a highly genomic instability involving both ploidy and structural rearrangements. One of the most frequent abnormalities is the t(4;14) (p16;q32) translocation, which occurs in approximately 15-20% of the patients. The translocation causes the simultaneous dysregulation of two potential oncogenes at the 4p16.3 locus, FGFR3 (Fibroblast Growth Factor Receptor 3) and MMSET/WHSC1 (Multiple Myeloma SET domain/Wolf-Hirschhorn Syndrome Candidate gene 1). The juxtaposition of endogenous promoters to powerful regulatory regions of the IGH locus (14q32) results in the association of MMSET with the intronic enhancer of IGH on der(4), whereas FGFR3 is dysregulated on der(14). MMSET codes for three putative isoforms, generated by mechanisms of alternative splicing: MMSET 647 (MMSET11), MMSET 1365 (MMSET24) and MMSET 584 (RE II–BP). The functions of these proteins are still unknown, but the presence of evolutionarily conserved domains such as SET, PHD, HMG suggest a possible involvement in chromatin remodelling and transcriptional repression mechanisms. The only experimental evidence is relative to the isoform RE II–BP, which binds a region on the promoter of IL-5 gene, RE–II (Response Element II), repressing its transcription. In order to investigate the transcriptional activity of MMSET isoforms, we constructed vectors expressing fusion proteins in which MMSET11, MMSET24 or RE–II BP were linked to the Gal4-DNA binding domain (amino acids 1–147). Immunofluorescence analyses of transfected 293T cells, using specific antibodies, showed the nuclear localisation of MMSET11 and MMSET24, whereas RE–II BP was localised in the cytoplasm. Co-transfections of the MMSET isoforms with a reporter gene (dual luciferase reporter assay
EXPRESSION OF ANNEXINS A2 AND A6 IN MULTIPLE MYELOMA: OPPOSITE FINDINGS BY IMMUNOHISTOCHEMISTRY AND GENE EXPRESSION PROFILING


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Among members of the Annexin family, Annexin-A2 (ANXA2) and Annexin-A6 (ANXA6) are involved in exocytosis and membrane trafficking in a variety of cells. Prompted by our interest in investigating the potential role of these two proteins in immunoglobulin secretion by plasma cells (PC), we decided to use the published micro-array gene expression datasets derived from multiple myeloma (MM) patients\(^1,2\) to get insights into the expression profile of ANXA6 and ANXA2 genes in PCs. Mining these datasets for the ANXA2 and ANXA6 genes led to the result that in a large fraction of PC samples ANXA2 was highly expressed and ANXA6 was not consistently expressed. To confirm at the protein level these mRNA findings, we performed immunohistochemical studies on B5-fixed paraffin-embedded bone marrow biopsies from 30 MM patients, that were immunostained with specific antibodies against ANXA2 (mouse monoclonal antibody, 1:200 dilution, Transduction Laboratories) and ANXA6 (goat polyclonal antibody, 1:800 dilution, Santa Cruz Biotechnology), using EDTA/microwave-based antigen retrieval and the APAAP technique. Notably, neoplastic PCs from all 30 samples showed an expression pattern opposite to that suggested by the microarray datasets, i.e. strong expression of ANXA6 protein (with ANXA6-negative erythroid cells serving as internal negative controls) and absence of ANXA2 protein (with ANXA2-positive endothelial and myeloid cells serving as internal positive controls). The specificity of ANXA6 immunostaining was further confirmed by Western blots of purified CD138\(^+\) PC lysates from two MM patients that, upon probing with the same antibody, showed a band of the expected molecular weight for ANXA6 (70 kDa). We next extended the immunohistochemical analysis of ANXA2 and ANXA6 expression also to non-neoplastic PCs in reactive lymph nodes (n=10) and palatine tonsils (n=5), obtaining identical results. These findings suggest that, if any role is played by ANXA2 and ANXA6 in immunoglobulin secretion from PCs, attention should be focused on ANXA6 rather than ANXA2. This is also in keeping with the involvement of ANXA6 in digestive enzymes secretion from pancreatic acinar cells through interaction with CRHSP-28/TPD52,\(^3\) an epithelial-associated protein that we recently found over-expressed in normal and neoplastic PCs as compared to the other B-cell subsets (Tiacci E and Falini B, unpublished observations). The discordant expression at the mRNA and protein levels of both ANXA2 and ANXA6 genes in PCs add to previous data on the discrepancy between transcriptional and translational level of gene expression reported in other settings.\(^4\) While recognizing the importance of microarray analysis in unraveling transcriptional signatures on a genome-wide basis, we suggest a word of caution when attempting to translate data from global mRNA-based profiles into informations about protein expression of single genes.

References

CD52 ANTIGEN EXPRESSED ON PRIMARY PLASMA CELLS AND MULTIPLE MYELOMA CELL LINES IS EFFICIENTLY TARGETED IN VIVO BY ALEMTUZUMAB

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Multiple myeloma (MM) is an incurable clonal plasma cell (PC) disorder. Although autologous stem cell transplantation improves survival, most patients relapse, and salvage therapy options remain limited. New treatments targeted to the malignant PCs are therefore needed. Alemtuzumab, a humanized monoclonal antibody to CD52 capable of destroying CD52+ cells by antibody-mediated cellular cytotoxicity as well as through complement fixation, is currently approved for therapy of chronic lymphocytic leukemia (CLL). In order to evaluate the therapeutic potential of alemtuzumab for myeloma patients, we examined CD52 expression on primary malignant marrow PCs as well as a panel of MM continuous cell lines (RPMI-8226, KMS-11, OPM-2, U-266, LP-1). In addition, the anti-myeloma activity of alemtuzumab was evaluated in vivo in a xenotransplant model of MM in nonobese diabetic/severe combined immunodeficient (NOD/SCID) mice. Primary PCs were enriched from the bone marrow of patients (n = 35) with MM according to CD138 expression. By using an immunomagnetic technique (Miltenyi Biotec, Germany, EU), highly purified CD138+ cells (median purity = 93%; median recovery = 55%) were obtained and further characterized by 3-color flow cytometry. On average, 63% of MM patients expressed CD52 on CD138-enriched cells, with a median of 50% PCs expressing CD52. Among our MM patients, 44% had CD52+ PC. Expression of CD52 was equally detected on CD138+CD45+ and CD138+CD45- PCs (p = 0.05). Analysis of CD52 expression on MM cell lines resulted in rather heterogeneous findings, with dim positivity on KMS-11, OPM-2 and LP-1, bright positivity on U-266, and negativity on RPMI-8226. CD52 expression as evaluated by flow cytometry was confirmed by quantitative PCR analysis of primary PCs and MM cell lines. The in vivo activity of alemtuzumab was evaluated in a xenotransplant model of MM in NOD/SCID mice. Mice were inoculated intravenously with KMS-11 cells (0.5×10^6 per mice) and were treated with alemtuzumab (3×1 mg/mouse, subcutaneously, days 4, 7, 9). Endpoint was mice survival. CD52-treated mice were compared to placebo-treated NOD/SCID mice. All placebo-treated mice (n = 18) died after a median survival of 55 days, whereas 44% of mice treated with alemtuzumab were alive with a median survival of 76 days (p = 0.0001 by log rank test). No mice experienced any apparent treatment-related toxicity. According to these data, we conclude that: (1) CD52 is expressed on the plasma cells of a significant proportion of MM patients; (2) alemtuzumab has a strong antitumor activity in vivo on CD52-positive MM cell lines; (4) alemtuzumab might have therapeutic potential in a subset of MM patients.

FREQUENCY AND PROGNOSTIC RELEVANCE OF T(4;14) IN PREVIOUSLY UNTREATED MULTIPLE MYELOMA (MM) PATIENTS RECEIVING EITHER SINGLE OR DOUBLE AUTOTRANSPANTS

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In patients with MM a number of recurrent translocations involving chromosome 14 at band q32 have been recently identified, the most common being the t(11;14) and the t(4;14). Both these chromosomal abnormalities are closely associated with particular presenting features of the disease and may help to identify patients at different risk of death. In particular, the t(11;14) predicts for good prognosis, whereas the t(4;14) has been reported to be an unfavorable prognostic feature. The t(4;14) has been described almost exclusively in MM patients, although its exact role in the pathogenesis of the disease has not fully elucidated. The translocation affects at least two potential oncogenes, MMSET on der(4) and Fibroblast Growth Factor Receptor 3 (FGFR3 on der (14); the role of two additional genes (TACC3 and LETM1) located near the breakpoint region on chromosome 4 has not yet been evaluated. In the present study we investigated the frequency and the prognostic relevance of the t(4;14) in a series of 63 patients with de novo MM, who were randomized to receive either a single (Tx-1) or a double (Tx-2) autotransplant as primary therapy for their disease. For this purpose we analyzed 1) the presence of t(4;14) by RT-PCR of the hybrid transcript between MMSET and the IgH locus; 2) the overexpression of FGFR3 by Real-time RT-PCR; 3)
the relationship between t(4;14), response to high-dose therapy and outcome of autotransplant(s). Overall, the t(4;14) was detected in 17/63 patients (27%), a value slightly higher than that reported by others. 13/17 patients had both MMSET/IgH fusion gene and FGFR3 overexpression, while 4 patients had MMSET/IgH but did not overexpress FGFR3. This finding further confirms the possible discrepancy between MMSET/IgH positivity and FGFR3 overexpression. Comparison between t(4;14)+ and t(4;14)− patients revealed that both groups were well balanced with respect to the most common presenting features of MM. In 36 patients, for whom material was available, FISH analysis for the detection of 13q deletion and/or monosomy was performed. Results showed that t(4;14)+ patients were more likely to carry also del(13) than t(4;14)− patients (46% vs. 29%, respectively). Among patients who attained stringently defined complete remission following either Tx-1 or Tx-2, t(4;14)− patients were 35%, as opposed to 60% of t(4;14)+ patients (p = 0.05, intention to treat). With a median follow-up of 45 months, no difference in overall (OS) was disclosed between t(4;14)− and t(4;14)+ patients, on the contrary the event free survival turned out significantly longer in patients without translocation. In summary, 27% of our MM patients carried the t(4;14). In this cohort of homogeneously treated patients, the t(4;14) predicted for lower response to high-dose therapy. Longer follow-up is required to assess the influence of this abnormalities on OS and EFS.

Funding: This work was supported by MURST COFIN 2002 and 2003, A. I. R. C., ATENEO 60% target projects, FIRB 2001, "Hairshow" A. I. L., "Fondazione del Monte di Bologna e Ravenna" grants, Fondazione Carissbo.

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**CO-63**

**GENE EXPRESSION PROFILING OF MULTIPLE MYELOMA REVEALS A ROLE OF CHROMOSOMAL TRANSLOCATIONS FOR THE DEFINITION OF DISTINCT ENTITIES OF MALIGNANCY**

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Multiple Myeloma (MM) is a neoplastic disorder of bone marrow plasma cells (PCs) characterized by a marked genetic heterogeneity and an extremely varied clinical course, including extra-medullary forms (plasma cell leukemia, PCL). MM may be preceded by a pre-malignant condition called monoclonal gammopathy of undetermined significance (MGUS). Chromosomal translocations involving the immunoglobulin heavy-chain locus (IGH) and a promiscuous array of partner loci represent early and frequent genetic aberrations in MM. To provide insights into the molecular pathogenesis of MM and to investigate the contribution of specific genetic lesions to the clinical variability of MM, we analysed the gene expression profiles of PCs isolated from 3 normal donors and 53 patients affected by different forms of plasma cell dyscrasias (8 MGUS, 39 MM and 6 PCL) by DNA microarrays representative for ~18,000 transcripts. Our results indicate that MM is characterised by highly heterogeneous phenotype at the transcriptional level, while MGUS samples can be distinguished from PCL and the majority of MM cases. A close correlation was found between distinct types of chromosomal translocations and specific gene expression profiles in MM. In particular, we identified a peculiar signature in cases with translocated MAF and MAFB genes, which involved the deregulation of MAF and MAFB genes, and aggressive clinical evolution who expressed a set of cancer germ line-specific antigens. This finding could have implications for patients’ classification and immunotherapy in MM. In general, our study supports the notion of a marked heterogeneity of MM and highlights the relevance of different genetic lesions. Furthermore, these data may provide insights into the role of distinct molecular pathways in myelomagenesis and contribute to the identification of novel molecules for targeted therapies.

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**CO-64**

**MULTIPLE MYELOMA: CHROMOSOME 13 ABNORMALITIES (DEL13) DETECTED BY FLUORESCENCE IN SITU/HYBRIDIZATION (FISH) AND CORRELATION WITH PLASMA CELL IMMUNOPHENOTYPE**


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Fluorescence *in situ* hybridization (FISH) is widely used for the detection of chromosomal abnormalities as an alternative to conventional cytogenetic techniques, in particular in hematological malignancies characterized by a low proliferative index such as most plasma cell disorders. In this study, interphase FISH has been employed to evaluate chromosomal abnormalities in monoclonal gammopathies. Between October 2002 and April 2004 bone marrow aspirates from 164 consecutive patients were evaluated for chromosome 13 deletion (del13). 147 were patients with multiple myeloma (MM), including 102 at diagnosis and 45 at relapse, 12 were monoclonal gammopathies of undetermined significance (MGUS) and 5 plasma cell leukemias (PCL). Bone marrow plasma cells were enriched using anti-CD138-coated magnetic beads (Miltenyi Biotech, UK) and the purity determined by flowcytometry with PE-conjugated-anti CD38 monoclonal antibody. The percentage of plasma cells exceeded 85% in all samples. Nuclei from fixed plasma cells were prepared for interphase FISH analysis using standard methods. Del13 was identified using LSI13RB1 probe (Vysis, UK). For each sample, 200 plasma cells were evaluated. Del13 was identified in 53% of MM, in 75% of MGUS and in 60% of PCL patients, respectively. The incidence of del13 was 58% and 48% in MM at diagnosis and at relapse, respectively. There was no significant difference in the prevalence of del13 according to age, bone marrow plasma cell infiltrations, serum β2-microglobulin, and C reactive protein levels, clinical stage and immunoglobulin isotype. Del13 was observed more frequently in female (56% vs 34% in non del13 patients, p=0.015) and in patients with lambda light chains (50% vs 26%, p=0.02); patients with del13 had more frequently serum monoclonal paraprotein concentration <1g/dL (42% vs 24.5, p=0.04). One MM patient who showed a biallelic loss of signal in 85% of purified plasma cells rapidly progressed to PCL. The immunophenotypic profile of bone marrow plasma cells has been assessed by evaluating the expression of CD38, CD138, CD56, CD45, CD40, CD19, CD20, CD52 and CD117. There was no statistically significant difference in the expression of these antigens between patients carrying del13 and patients without chromosome 13 abnormalities. Patients with del13 were more frequently negative for CD45 and CD56 than patients without del13 (66% vs 50%, p=0.07; 25% vs 15%, p=0.1). Though not statistically significant, an increase in the expression of surface monoclonal immunoglobulins was observed in del13 patients (44% vs 26% in non del13 patients, p=0.08). This characteristic has been previously associated with poor prognosis by our group and other Authors. We also noted that patients with more than 50% of plasma cells carrying del13 had a significantly higher bone marrow plasma cell infiltrations (19% vs 6%, p = 0.008). Due to the short follow-up, no definite conclusion can yet be drawn on the prognostic relevance of del13. However, our preliminary data appear consistent with the observations reported in other studies. Evaluation of del13 by FISH should be mandatory in the design of future clinical trials to determine its real role as prognostic factor.
C-KIT point mutations are not a rare event in core binding factor (CBF) leukaemia; in this setting we previously reported the presence of both kinase or extracellular-juxtamembrane mutations in 46% of patients. To investigate the prognostic impact of c-KIT gene mutations, we attempted to correlate the presence of the mutation with the WBC count, the level of the WBC-index (WBC × [% BM blasts/100]) and the clinical outcome, in 45 CBF-AML patients. The 45 patients, M:F 37/8, had a median age of 46 years (range 16-88); cytogenetic showed a t(8;21) in 27 cases and inv(16) in the remaining 18. On the basis of a genomic DNA sequencing of c-KIT gene for exon 2, 8, 10, 11, 17 mutations, 19 patients (42%), categorized in the KIT mutated group (KIT+), showed a mutation in either exon 8 (n = 4), exon 17 (n = 13), exon 10 (n = 1) and exon 11 (n = 1). The remaining 26 patients, in which the mutation was not detected, were categorized in the KIT non mutated group (KIT−). Considering the whole group of patients, we found a mean WBC count of 57.7 × 10^9/L vs 16.9 × 10^9/L (p = 0.004) and a mean WBC-index of 40.29 vs 12.56 (p = 0.019), for KIT+ and KIT- respectively. Thirty-six patients (age < 60 years) were considered evaluable for clinical response. At a median follow-up of 20 months (range 6-103), we recorded for KIT+ and KIT- respectively: CR incidence of 93.7% (15/16) vs 100% (7/20) p = NS; Relapse Incidence 81.2% (13/16) vs 35% (7/20) p = 0.015; OS 25% (4/16) vs 75% (15/20), p = 0.008 and DFS 12.5% (2/16) vs 75% (13/20) p = 0.005. In conclusion, this study confirm the correlation between c-KIT mutational status with high WBC count and WBC-index in CBF-AML patients as previously reported (Leukemia 2003; 17: 471-472). Furthermore our data indicate a statistical correlation between the presence of KIT mutations and both the OS and DFS.
PO-003
DETECTION OF DIFFERENT MLL REARRANGEMENTS IN ADULT ACUTE LEUKEMIA BY MULTIPLEX PCR AND FLUORESCENT CAPILLARY ELECTROPHORESIS

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Background: Chromosome11q23/MLL (mixed lineage leukemia) rearrangements characterize a subgroup of acute leukemias with intermediate prognosis. Their clinical significance is strongly dependent on the different fusion gene involved, making important an accurate identification of the specific chimeric gene. We have developed two parallel triplex PCR for the identification of the commonest types of MLL translocations in hematologic malignancies of adults, like t(4;11), t(6;11), t(9;11), t(10;11) and t(11;19).

Methods: Total RNA from cell samples was reverse transcribed. Amplification of MLL and related fusion genes was obtained using the primers described by Andersson (2001), but we have paired them differently and changed the amplification conditions. Mix1 contained a unique 5’MLL-specific external primer in combination with 3’ primers specific for AF4, AF9 and AF10 genes, Mix2 contained the 5’MLL primer used for the Mix1, in combination with a set of 3’ primers specific for AF6, ELL and ENL genes. Both Mix1a and Mix2a contained the internal 5’MLL primer and a set of internal 3’ primers for the detection respectively of AF4, AF9, AF10 fusion genes and of AF6, ELL and ENL genes. Each assay was carried out with a negative control (cDNA templates were from healthy subjects and from CML and AML M3 patients) and a reaction control (amplification of a mixture without cDNA).

Positive controls from previously studied patients were available for AF4, AF9 and AF10 genes. If amplification fragments of appropriate dimensions range are obtained after triplex PCR, split-out reactions were performed. To this were used the common 5’MLL internal and external primer pairs labeled with two different fluorochromes and the two 3’ primers specific for the chimeric gene previously identified. For a more accurate size identification, PCR products were subjected to capillary electrophoresis and analyzed by gene scanning in an automatic DNA sequencer ABI Prism 310.

Results: A total of 8 acute lymphoblastic leukemias and 22 acute myeloid leukemias, without BCR/ABL and PML/RARA rearrangements, were analyzed. Our paired triplex PCR was able to detect amplification products of MLL/AF4, MLL/AF9, MLL/AF10 in all three known patients. No false positives were detected in 10 normal subjects, 20 CML and M3 patients. Conclusions: The multiplex PCR method described here allowed an accurate and less time consuming identification of at least three clinically important translocations involving MLL. Validation of this methodology is underway for AF6, ELL and ENL genes. Because of the variability of the breakpoints of all the tested genes, the second step of fluorescence-based analysis allows a more accurate identification of the product amplification size and could be potentially useful to monitor minimal residual disease.

PO-004
CONTINUOUS SEQUENTIAL INFUSION OF FLUDARABINE AND CYTARABINE FOR ELDERLY PATIENTS WITH ACUTE MYELOID LEUKEMIA: A PHASE II STUDY

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The prognosis of acute myeloid leukemia (AML) in elderly patients is still poor, therefore new approaches are needed. We previously demonstrated the feasibility of a regimen based on the combination of fludarabine (F) with cytarabine (ARA-C) given as continuous sequential infusion (CI–FLA). In particular, F was administered at a loading dose of 10 mg/m² over 15 min at day 0 followed by continuous infusion (CI) of 20 mg/m²/24 hours for 72 hours; ARA-C was given at a loading dose of 390 mg/m² over 15 min three hours and half after FAMP and then as CI over 96 hours at 1440 mg/m²/24 hours. G-CSF was added at day +15 at a dose of 5 µg/kg. Patients achieving complete remission (CR) were programmed to receive an additional identical course of CI–FLA. However, after the first 20 patients consolidation was reduced by one day due to excessive toxicity. Following consolidation, G-CSF at 10 µg/kg was given from day 15 with the aim of shortening neutropenia and mobilizing CD34+ cells. Toxicity was recorded according to WHO criteria. Here we report our updated experience on a larger cohort in order to evaluate results on disease free (DFS) and overall survival (OS). From December 2001 60 untreated patients aged over 60 years with non M3 AML have been accrued in two different Institutions. The median age was 69 years (range 61-82). In 22 patients (37%) a previously diagnosed myelodysplastic syndrome (MDS) preceded the onset of AML. Cytogenetic analysis showed normal karyotype in 29 patients (48%), complex karyotype or other unfavorable chromosomal abnormalities in 21 (35%), and no mitosis in 10 (17%). Of note, 50 patients (83%) were affected by concomitant disease requiring specific treatment. Overall, 39 patients achieved CR (65%), all following
one course of CI-FLA. There were 10 induction deaths (17%), while 11 patients were primary refractory (18%). The median number of days to neutrophil >1.0x10^9/L and platelet >20x10^9/L recovery was 19 (7–34) and 19 (9–38), respectively. Patients needed a median of 3 platelet units (1–13) and 6 blood units (1–22), respectively. All patients experienced severe pancytopenia requiring broad spectrum empiric antibiotic therapy. Induction deaths were due to infectious episodes occurred during the aplastic phase (n=8) and cerebral hemorrhage (n=2). Among remitters, 33 out of 39 (85%) received the programmed consolidation course, while in 6 cases therapy was discontinued due to unresolved infection (n=2), persistent thrombocytopenia (n=2), cerebral hemorrhage occurred after CR attainment (n=1), patient’s desire (n=1). Twenty-nine patients were monitored for the mobilization of CD34+ cells, collection being successful in 20/29 patients were monitorized for the mobilization of CD34+ cells, collection being successful in 20/29 (69%). The median number of CD34+ cells collected was 6.5x10^9/kg (range 2.4–60.3), the median number of apheresis being 2 (1–3). Overall, 16 patients have received autologous stem cell transplantation. At the time of writing 26 patients are alive: 20 are in continuous CR after a median follow up of 8 months (range 2–27), 4 are alive with refractory and 2 with relapsed disease, respectively. In conclusion, these data confirm the feasibility of CI-FLA and the extremely encouraging results in terms of CR achievement and CD34+ cell collection. Data on DFS and OS are promising, but a longer follow up is needed.

**PO-005**

FREQUENT ABERANT BRCA1 METHYLATION IN SECONDARY ACUTE MYELOID LEUKEMIA


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Aberrant DNA methylation has been observed in many human tumors, in particular in the CpG islands of tumor suppressor genes. BRCA1 is a tumor suppressor gene that encodes a 1863 amino acid protein that is important for regulation of cell proliferation, participation in DNA repair/recombination processes related to the maintenance of genomic integrity, induction of apoptosis in damaged cells and regulation of transcription. Recent data reveal that promoter hypermethylation is frequently associated with BRCA1 inactivation in non-inherited breast and ovarian carcinomas. Using a methylation-specific PCR, we studied BRCA1 promoter hypermethylation in 126 patients (60 females, 66 males, median age 62 years, range 16–85 years) with acute myeloid leukemia (AML). BRCA1 was hypermethylated in 68/126 (54%) AML samples. The functional role of BRCA1 hypermethylation was confirmed in 46 patients whose RNA was available, where hypermethylation correlated to loss of expression. Eight of 22 methylated samples were RT-PCR-negative for BRCA1 expression, while only 1 of 24 unmethylated samples were PCR-negative (p=0.009). BRCA1 promoter hypermethylation was more frequent in females than in males (40/60, 67% versus 28/66, 42%, p=0.008, OR=2.7, 95% C. I. =1.3–5.6). Twenty-five patients had a leukemia secondary to a previous malignancy (s-AML), treated using chemo- and/or radiotherapy in 19 patients. A significant association between s-AML and BRCA1 promoter hypermethylation was found (19/25, 71% versus 49/101, 49%, p=0.01, OR=3.4, 95% C. I. =1.2–9.1), in particular when restricting the analysis to therapy-related AML (16/19, 84% versus 52/107, 49%, p=0.005, OR=5.6, C. I. =1.5–20.5). Interestingly, all 6 patients (100%) treated for a previous breast cancer were hypermethylated for BRCA1. Patients with white blood counts higher that 30 x 10^9/L also had a significantly higher frequency of BRCA1 hypermethylation, when compared to patients with white blood counts lower than 30 x 10^9/L (37/78, 47% versus 41/78, 53%, p=0.05, OR=2.7, C. I. 1.1–7.0). No differences were noted when looking at age, blast-cells percentage in the bone marrow, karytype, FAB-subtype and response to treatment. Since aberrant hypermethylation of different genes has been reported in therapy-related AML, BRCA-1 hypermethylation could be one of the transformation pathways in s-AML, related to inefficient DNA repair, following antiblastic treatment. On the other hand, this study points to a possible link between genetic instability related to BRCA1 inactivation in non-inherited breast cancer and secondary leukemic transformation.

**PO-006**

MINIMALLY DIFFERENTIATED ACUTE MYELOID LEUKEMIA (AML-M0) EXPRESSES HIGH LEVELS OF BCL-2 AND LOW LEVELS OF MDR1 PROTEIN


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Two-hundred and 56 consecutive cases of de novo AML were studied for the expression of bcl-2 and MDR1. Within this series, 28 (11%) patients with AML-M0 were identified. The results were expressed as relative mean fluorescence intensity (rMFI) and an index which equals the product of the percentage of positive cells and rMFI. Bcl-2 MFI was significantly higher in M0 (median 24.6, range 10.3–107), M6 (median 23.4, range 4.2–83) and M1 (median 18.2,
range 3.4-87.4) subtypes \( (p = 0.00001) \), whereas MDR1 MFI was higher in M5 (median 11.4, range 1.6-69.1) and M4 (median 6.9, range 1-56), compared with the other FAB subgroups \( (p = 0.00001) \); in AML-M0, the value of MDR1 MFI was the lowest (median 1.6, range 0.9-20). Conversely, we observed the highest value of bcl-2 index in M0 (median 1732, range 241-10025), M6 (median 1606, range 290-3616) and M1 (median 1437, range 146-7604) \( (p = 0.00001) \), whereas MDR1 index was the highest in M5 (median 851, range 6-4975) and M4 (median 397, range 3-3920). This first step of analysis demonstrates that each FAB subgroup expresses bcl-2 and MDR1 in a quite heterogeneous amount, so that a given quantitative combination of the two proteins may be peculiar for a specific AML subtype. Based on this, we designed a ratio dividing the value of bcl-2 index by that of MDR1 (bcl-2:MDR1 ratio); the bcl-2:MDR1 ratio was then calculated for each FAB subgroup. Bcl-2:MDR1 ratio was very high in AML-M0 \( (p = 0.00001) \), therefore, AML-M0 qualifies as a true distinct entity due to a predominant expression of bcl-2, whereas the remaining FAB subtypes express variable level of both bcl-2 and MDR1 proteins, or MDR1 protein alone. To further investigate these aspects, we also calculated the CD34 and CD71 index; the latter was used as an indirect tool to explore the cell-cycle status. The highest value of CD34 index was found in AML-M0 (median 6076, range 1-95100) \( (p = 0.00001) \); in line with this, we also observed a direct correlation between CD34 index and bcl-2:MDR1 ratio \( (r = 0.37, p = 0.00001) \). Finally, the value of CD71 index was very low in AML-M0 (median 80.5, range 1.7-10025) \( (p = 0.00001) \), consistent with a lack of proliferative activity. From a clinical point of view, the median age of patients with AML-M0 was 64 years \( (\text{range} \ 30-78) \), 12 were females and 16 males. The presenting median white blood cell count (WBCc) was 7.4×10^9/L \( (\text{range} \ 0.7-122×10^9/L) \) in patients with non-M0 AML was 62 \( (\text{range} \ 18-78) \), 104 females and 124 males, presenting median WBCc 20×10^9/L \( (\text{range} \ 1.1-390×10^9/L) \); the difference in WBCc between AML-M0 and non-M0 AML patients was statistically significant \( (p = 0.006) \). The CR rate for AML-M0 and non-M0 AML group was 43% \( (12/28) \) and 66% \( (153/228) \), respectively \( (p = 0.02) \). Finally, AML-M0 had a shorter duration of survival \( (p = 0.030) \) and disease free survival \( (p = 0.06) \). In conclusion, our results suggest that in AML-M0 bcl-2 is over-expressed and may represent a key-protein involved in the chemo-resistance process. This has therapeutic implications since the use of apoptosis inducers may be indicated for improving treatment outcome in this subgroup.

Internal tandem duplications (ITD) and mutations at codon Asp835 of FLT3 (fms-like tyrosine kinase 3) are the most frequent aberrations in acute myeloid leukemia (AML) and are a powerful negative prognostic factor. Aberrations of FLT3 are not associated with karyotypic abnormalities, and are especially helpful in determining prognosis in patients with a normal karyotype who are at intermediate risk. We have previously shown the prognostic impact of deletions of 2 enzymes of the xenobiotic detoxification pathway, GSTT1 and GSTM1, for the outcome in patients with AML particularly in patients with an intermediate risk karyotype (Voso et al., Blood 2002; 100:2703). We were therefore interested on possible interactions between defects in the xenobiotic detoxification pathway of glutathione S-transferase (GSTM1, GSTT1, GSTP1) and cytochrome p450 (CYP1A1*2A, *2B and *4 alleles) and FLT3 aberrations. Study population included 170 patients with AML (79 females, 91 males), of a median age of 62 years \( (\text{range} \ 16-87) \). The prevalence of FLT3-ITD in our patients was 15.3% \( (26/170) \), while that of Asp835 was 4% \( (3/74) \). A significant association between FLT3-ITD and age or gender were found. Patients with a complex karyotype had a lower incidence of FLT3-ITD than those with normal karyotype or a balanced translocation. Patients with AML and associated myelodysplasia or leukemia related to chemotherapy for a previous malignancy also had a lower incidence of FLT3-ITD, when compared to patients without MDS or with a de novo AML \( (0/37, 0\% \text{ versus} 26/122, 21.3\% \) versus 0/37, 0% versus 26/122, 21.3% \( p = 0.000, \text{ O. R. 6.8, 95\% C. I. 2.4-19.1} \). No associations between FLT3-ITD and age or gender were found. Patients with a complex karyotype had a lower incidence of FLT3-ITD than those with normal karyotype or a balanced translocation. Patients with AML and associated myelodysplasia or leukemia related to chemotherapy for a previous malignancy also had a lower incidence of FLT3-ITD, when compared to patients without MDS or with a de novo AML \( (0/37, 0\% \text{ versus} 26/122, 21.3\% \) versus 0/37, 0% versus 26/122, 21.3% \( p = 0.000, \text{ O. R. 6.8, 95\% C. I. 2.4-19.1} \). This indicates that leukemias following myelodysplasia or a previous chemotherapy may follow pathways different from those due to FLT3 activation. Looking at GST, the GSTM1 null genotype was detected in 26% of AML patients and the GSTT1 null genotype in 26% of patients, while 12.5% of patients had a double-null genotype. The prevalence of CYP1A1*2A, *2B and *4 alleles was 8.1%, 7.7% and 21%, respectively. We have previously reported patients’ characteristics associat-
ed to GST deletions and CYP1A1 alleles. The prevalence of the GSTP1Val mutation was studied in 151 patients. Eleven patient (7.3%) were homozygous for the Val/Val mutated genotype, 47 (31.1%) had a heterozygous Ile/Val GSTP1 genotype, and 93 (61.6%) had a normal Ile/Ile GSTP1 genotype. No associations between GSTP1 allelic variants and patients' characteristics or prognosis were found. In 112 patients receiving standard induction therapy, an association between FLT3-ITD-positivity and toxic deaths, shorter disease-free and overall survival was found. Five of 11 toxic deaths (45%) versus 17 of the remaining 101 patients (16.8%) were ITD-positive, \( p = 0.006, \text{OR} 4.1, 95\% \text{CI} 1.1-15 \). The median DFS and OS of patients with positive FLT3-ITD were 4.8 and 7.2 months, compared to 13.7 and 16.8 months in patients without FLT3-ITD, \( p = 0.03 \) and \( p = 0.02 \), respectively. As previously shown, and with longer follow-up, the presence of one GST deletion (T1 and/or M1) was not associated to toxic deaths, but confirmed to be a negative prognostic factor for survival (median DFS and OS for patients with GST deletions 8.8 and 13.2 months versus 17.3 months and 29.4 months in patients without deletions, \( p = 0.01, p = 0.02 \), respectively). CYP1A1*2A also identified a prognostically unfavorable group of patients (median DFS 5.6 versus 14 months, \( p = 0.002 \), and median OS 10.5 versus 17 months, \( p = 0.006 \). The multivariate analysis using the Cox regression model confirmed that the presence of GSTM1 and/or GSTT1 deletions, CYP1A1*2A and FLT3-ITD were independent prognostic factors for survival. These data confirm that the individual detoxification profile may be a useful prognostic marker, independent of FLT3-ITD, in patients with AML.

**PO-008**

**METABOLIZING ENZYMES AND ACUTE LEUKEMIA: ROLE OF GENETIC POLYMORPHISM**


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Everyone has a unique combination of polymorphic traits that modify susceptibility and response to drugs, chemicals and carcinogenic exposures. Toxicants to which an individual is exposed are bio transformed and eliminated from the body after metabolic conversion mediated by Phase I and Phase II xenobiotic-metabolizing enzymes. Phase I enzymes catalyze hydroxylation, reduction and oxidation reactions of xenobiotics (carcinogens/drugs), often converting them into more active or toxic compounds. Phase II enzymes catalyze conjugation reactions (glucuronidation, acetylation, methylation), thereby converting the metabolites into non-reactive, water-soluble products that are eliminated from the organism.

Genetic polymorphism in the genes encoding for drug-metabolizing enzymes, underlying the variation in enzyme activity, can modify individual susceptibility to cancers as well as the response to therapy. We studied different common polymorphisms in the genes encoding for cytochromes CYP3A4 and CYP2E1, glutathione S-transferases (GST-M1), NAD(P)H: quinone oxidoreductase (NQO1) in 79 patients affected by acute leukaemia (AL) and 75 healthy control. A total of 7 allelic variants were evaluated, in particular the NULL genotype for GST-M1, three CYP3A4 single nucleotide polymorphisms (SNPs): A-392G, T15615C and T20072C, two CYP2E1 SNP: C1053T and G1293C, SNP C609T for NQO1. Sequences and selected polymorphisms were retrieved from www.ncbi.nlm.gov web-site. Genotypes were examined by a polymerase chain reaction (PCR) approach. Briefly, sequences of interest including the polymorphic sites were amplified by a first PCR followed by a second single base extension reaction using oligonucleotides appropriately designed to detect the SNPs and fluorescently labelled dideoxynucleotide. The latter reaction was performed using the SNAPshot assay (Applied Biosystem). SNP-PCR products were detected by capillary electrophoresis on a DNA Genetic Analyzer (ABI PRISM 3100 Applied Biosystem, CA). Collected data were visualized on a fluorescent histogram and analyzed by using ABI GeneScan software (Applied Biosystem, CA, USA). The analyzed AL patients showed an incidence of CYP3A4, CYP2E1 and GST-M1 genotypes similar to that found in the control group. In fact we found 12.6% heterozygosity for CYP3A4 and 10% in control group; a frequency of CYP2E1*5 allele of 17.7% in AL group and 20% in control group; a frequency of 38% of GST-M1 NULL in AL group and of 37% in control group. Furthermore we found only significant different distribution of NQO1 allele 609T in AL group (44%) versus control group (27%) \( \chi^2 \text{ test of Pearson} = 5.8 \). Our data show similar conclusions for some respect to those reached in previous investigations while identifying a different frequency of NQO1 609T genotype among tumors. We realize more patients need to be genotyped and other genes involved in the study to support solid conclusions. A number of studies have investigated drug metabolizing enzymes polymorphisms but their analysis was restricted to evaluate single or a limited number of SNPs. In our opinion the best approach is to extend the analysis to many candidates in order to represent a most comprehensive allelotype profile allowing for further, more refined associations between xenobiotic metabolizing pathways, the risk of cancer and response to therapy.
Constitutive activation of the MEK/ERK pathway is frequently observed in primary acute myelogenous leukemia (AML) samples, but not in normal CD34+ progenitors. ERK promotes cell growth and viability of leukemic cells, and its constitutive activation is an independent prognostic factor for survival in AML patients. We observed that the specific MEK inhibitor CI-1040 impaired cell growth and survival, abrogating clonogenicity of leukemic cells. No data is available on the effects of MEK inhibitors on primary relapsed/refractory AML cells. We applied a flow cytometry technique to quantitate the expression of phosphorylated ERK (p-ERK) in AML samples and normal CD34+ progenitor cells, and evaluated the in vitro effects of CI-1040. We analyzed p-ERK levels in bone marrow or peripheral blood samples from 42 primary AML patients (16 samples collected from AML at diagnosis and 26 from relapsed/refractory AML). Highly purified normal bone marrow and G-CSF mobilized CD34+ progenitors were collected and used as controls. ERK activation was analyzed using a monoclonal antibody specific for p-ERK and Kolmogorov-Smirnov statistics. Results were expressed as D values (D), which allows the objective quantitation of differences in fluorescence intensity. Samples with D > 0.1 were considered positive. In addition, we evaluated changes of p-ERK induced by short-term cell culture (up to 24h) in FCS (10%)-containing medium in the presence or absence of MEK inhibitor CI-1040 (3uM). Cell viability and phosphatidylserine externalization were determined. Normal bone marrow CD34+ cells at most weakly expressed p-ERK (D=0.07±0.035), while G-CSF mobilized CD34+ cells exhibited markedly levels of p-ERK (D=0.56±0.038). Likewise, marked p-ERK levels were found in 83.3% of AML samples, with a mean D value equal to 0.32±0.04. No differences between samples at diagnosis and relapsed/refractory samples with respect to p-ERK levels were observed: D values were 0.36±0.06 vs. 0.30±0.05 (p=n. s.). Next, we examined effects of the specific MEK inhibitor CI-1040. In AML cell lines with constitutive ERK phosphorylation, CI-1040 completely inhibited ERK activation. In primary AML, a statistically significant decrease in p-ERK levels were detected after a 24 hour incubation with CI-1040 D=0.46±0.04 to D=0.31±0.04 (p=0.00027). Newly diagnosis and relapsed/refractory samples were equally sensitive to the inhibitory effects of CI-1040. CI-1040 did not induce apoptosis compared to vehicle in all but one samples examined. In conclusion, the increase in p-ERK levels that were detected in mobilized normal circulating CD34+ cells was likely due to activation by G-CSF. In contrast, high levels of p-ERK observed in the majority of primary AML samples suggest a disregulation of the MEK/ERK pathway, which is apparently independent of disease stage. CI-1040 inhibited ERK activation in vitro without inducing apoptosis. Consequently, MEK inhibitors may be more effective in combination with cytotoxic agents for the therapeutic modulation of AML. Our previous data have suggested striking synergism with apoptosis modulators.
tion until optimal VPA plasma levels (80-110 µg/mL). RA (Vesnand [Roche]) at the dosage of 45 mg/m² p. o. /d, divided in two administrations, was added at optimal VPA plasma levels or at day 14 to day 28. Peripheral blood and/or bone marrow samples were collected at day 0, 3, 7, 14, 21, 28 for the evaluation of histone acetylation and for morphologic, immunophenotypic, cytogenetic, and molecular studies. Four patients had a history of MDS, three patients had a FAB M0, M1 and M2 de novo AMLs, while the remaining case was a myeloid blast crisis (FAB M0) from a Ph+ve CML. Of the other seven patients, one had normal karyotype, one a pseudodiploid [der(12)], one a hyperdiploid (+8) K, one a complex K with a 7q-alteration while in the three remaining cases the karyotype is not available. Pre-treatment leukemic infiltration ranged from 22% to 95%. VPA plasma level >60 g/mL was reached between 8 to 28 days (median 14.5 days). In three patients VPA-RA treatment induced hyperleukocytosis (> 50×10⁹/L) at day 16, 21 and 24, respectively, treated with chemotherapy (HU in two cases and low dose Ara-C in 1 case). Hematological improvement (50% decrease in packed red blood cell or platelet transfusion requirement) was observed in one case while a stable disease and disease progression were observed in five and two cases, respectively. All patients showed features of myeloid-monocytic and/or eritroid differentiation of the leukemic clone, as revealed by morphologic, cytochemical, immunophenotypic analyses and Q-RT-PCR of myeloid gene expression (GATA 1, MPO, CSF2R, etc.). High degree of myeloid differentiation correlated with early achievement of therapeutic VPA plasma levels and histone hyper-acetylation, as measured by immunocytochemistry and immunoblotting using antiacetylated histone H3 antibodies. Finally, differentiation of the leukemic clone was proven by FISH analysis showing the presence of the +8 and 7q- in maturing elements in patients whose leukemia blasts carried these cytogenetic lesions. VPA-RA combinational is a well tolerated treatment that induce phenotypic changes of the leukemic clone through chromatin remodelling. Further studies are needed to optimise this regimen with the aim of improving clinical response in leukemia patients.

**PO-011**

**USE OF MYLOTARG IN ELDERLY PATIENT WITH RELAPSED ACUTE MYELOID LEUKEMIA: EFFICACY AND TOXICITY**

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Acute myeloid leukemia (AML) in the elderly is complicated not only by comorbidities but also by the high prevalence of poor prognosis markers. Relapse represents the main adverse prognostic factor in patients with AML. Mylotarg (gemtuzumab ozogamicin, CMA-676; Wyeth-Ayerst Laboratories, Philadelphia, PA, USA) has recently been approved for the treatment of CD33+ acute myeloid leukemia in elderly relapsed patients. To investigate the efficacy and toxicity of Mylotarg, we report the case of a 71-year-old male who was admitted in our department of Hematology in November 2003 for fourth relapse of acute myeloid leukemia. Bone marrow aspiration confirmed the presence of 60% of blasts cells. Blasts immunophenotype in bone marrow showed CD33, CD13, CD117 and MPO positivity. Cytogenetic study on bone marrow showed normal cariotype. The patient had previously received several cycles of chemotherapy (Anthracycline, ara-c, fludarabine) achieving complete but temporary response (CR3 duration < 6 months). Since the patient had received the maximum dose tolerated of anthracycline, the treatment plan included mylotarg (6 mg/m² by i. v. infusion over 2 hours for two successive cycles on day 1 and 21) and ara-c (1.5 g/m² on day 3–5). Patient achieved complete remission with incomplete platelet recovery on day +52. Except grade 4 myelosuppression (according to WHO score), although heavy pretreated, the patient no experienced FUO sepsis, hyperbilirubinemia, VOD, nephrotoxicity and mucositis. Actually he is undergoing periodical follow up to evaluate by flow-cytometric analysis of bone marrow the rate of CD 117 and CD33 positivity and re-start at once the Mylotarg treatment without Ara-C. In conclusion this salvage regimen could be able to induce CR in this subset of poor prognosis AML patients, without significant toxicity. A longer follow up is needed to evaluate the efficacy.

**PO-012**

**TOXICITY AND EFFICACY OF AUTOLOGOUS STEM CELL TRANSPLANTATION IN ELDERLY PATIENTS WITH ACUTE MYELOID LEUKEMIA**

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Outcome of acute myeloid leukemia (AML) in patients aged >60 years is characterized by short duration of remission with less than 25% probability of remaining disease-free after 4 years (Buchner, Best Practice and Research, 2001). Aim of this study was to evaluate the toxicity and the efficacy of auto-
logy stem cell transplantation (ASCT) using HD-Busulfan as conditioning regimen in elderly patients with AML. Patients and methods. Between December 1997 to January 2004, 11 patients with diagnosis of de novo AML (4 cases) or MDS-AML (7 cases), median age 62 years (range 59–66), F:M = 5:6, were enrolled in this study. According to cytogenetic pattern, 2 patients were categorized in the high risk group and the remaining 9 patients in the intermediate risk. At the enrolment 10 patients were in first complete remission (CR) and one patient in second CR. The preparative regimen was Busulfan 16 mg/kg. Leukocytes and neutrophil recovery was accelerated by CsF administration in all cases. Patients received a median of 8x10^6/kg (range 4,12–16,1) unmanipulated PBSC, harvested after completion of the second consolidation course. Results. All patients were evaluated for side-effects, duration of chemotherapy-induced cytopenia and infections. The median time to reach a neutrophil count >500/µL was 11 days (range 8–16); a platelet count >20000/µL and >50000/µL were observed after a median of 11 days (range 0–34) and 13 days (range 0–46, respectively. CsF was administrated for a median time of 10 days. We did not record toxic deaths. Four patient experienced a WHO grade I-II liver toxicity. Two patients with severe mucositis required a continuous infusion of narcotics for pain control. During neutropenia we recorded 4 episodes of FUO, 1 pneumonitis and 1 sepsis (Staphylococcus epidermidis). With a median follow-up of 9 months from ASCT (range 3–16), the relapse incidence was 73% (8/11 patients), the overall survival 45% (5/11 patients) and the disease free survival 27% (3/11); at the present time only 3 patients are in continuous CR, 3, 4 and 12 months after ASCT. Conclusions. These results indicate that HD-Busulfan as conditioning regimen has good tolerability and mild toxicity, but poor efficacy in obtaining long-term disease-free survival in elderly AML patients submitted to ASCT.

PO-013
IMPACT OF BONE MARROW AND INFUSED RESIDUAL LEUKEMIC CELLS ON RELAPSE AFTER AUTOLOGOUS BONE MARROW TRANSPLANTATION IN ACUTE MYELOID LEUKEMIA PATIENTS


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Flow cytometry represent a method to detect minimal residual disease (MRD) in the vast majority of acute myeloid leukemia (AML) patients. The present study was designed to determine to what extent autologous stem cell transplantation (ASCT) might impact on the level of MRD in 31 patients enrolled onto intensive chemotherapy protocols EORTC/GIMEMA AML10/12 (induction, consolidation, ASCT). Furthermore the same immunologic fingerprint has been used to trace the amount of residual leukemic cells in the graft of 12 patients receiving peripheral blood stem cell (PBSC). By using a threshold of 3.5x10^4 bone marrow leukemic cells, 12 out of 31 patients (39%) were MRD+ before ASCT whereas the remaining 19 (61%) were MRD-. Within a median time of 7 months from ASCT all of the 12 patients in the MRD+ situation had a relapse, whereas 5 of 19 pts (26%) who were MRD- before the transplant relapsed after ASCT, within a median time of 11 months. The difference in relapse rate between the MRD+ and MRD- group was highly significant (p<0.001); the multivariate analysis confirmed the association between a MRD+ status before ASCT and high frequency of relapse (p = 0.0014). In 12 patients, using the same flow cytometric method, we were able to determine the amount of residual leukemic cells in the harvest. A mean of 11.1x10^6/kg residual leukemic cells (range 1–37.5) has been reinfused at the moment of the transplant. There was no correlation with CD34+ cells harvested. Selection of different thresholds of reinfused leukemic cells, did not allow groups with a higher risk of relapse to be identified. We conclude that 1) in patients with high levels of MRD, the residual leukemia is not eradicated by ASCT; 2) the amount of the leukemic residual cells infused with the transplant has no defined role in determining leukemic relapse after ASCT; 3) the prognostic role of in vivo MRD compared to residual leukemic cells reinfused with the autologous graft needs to be investigated on a larger number of patients.

PO-014
USE OF DEFIBRITUDE IN THE PROPHYLAXIS OF VOD IN FOUR PATIENTS AFFECTED BY RELAPSING OR CHEMOREFRACTORY AML TREATED WITH GEMTUBZAM OZOGAMICYN (MYLOTARG)


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Gemtuzumab Ozogamicin (GO, Mylotarg) is a recombinant humanized anti-CD33 monoclonal IgG4 antibody that delivers the potent cytotoxin, Calicheamicin, into CD33 expressing cells. The CD33 antigen is expressed on blast cells in 80% to 90% of acute myeloid leukemias (AML) but it is not expressed on pluripotent hematopoietic stem cells or on non-hematologic cells. Myelotarg is today a novel and
Defibrotide, a strong inhibitor of platelet aggregation and an inducer of vessel dilatation, without affecting the coagulation. Therefore, it prevents ischemic and thrombotic events that are both present in VOD. We report our experience on 4 patients treated with Mylotarg regimen and Defibrotide prophylaxis. Four consecutive patients (3 females and 1 male, median age: 55 years, range 38–70 years) with CD33+ AML were admitted between October 2003 and April 2004. They were affected by relapsed (older patients) or refractory (younger patients) AMLs. They received Mylotarg at the dose of 6 milligrams/m², day 1 and 14 and Defibrotide prophylaxis, 10 milligrams/kilo/day intravenously, from day -1 to day +30. Before GO therapy Bilirubin, AST and ALT values were in normal range. VOD was never observed in our series, but 2 patients have developed grade 2 hypertransaminasemia/hyperbilirubinemia. Another patients showed on day +13 abdominal pain and transient hypertransaminasemia/hyperbilirubinemia that disappeared after 5 days. An abdomen US demonstrated the presence of gallbladder stones. The remaining patient showed no toxicity signs. Although GO induced severe thrombocytopenia it was never observed an increase of the incidence of haemorrhage signs. On the other hand, Defibrotide administration was well tolerated and it was never observed any toxicity sign during the long-lasting administration. GO induced a PR in the two patients who were resistant to the previous treatments. Moreover, the other two patients achieved a CR and a PR, respectively (Table 1). Two patients died four and six months after therapy, respectively. We have not found death due to therapy toxicity. Conclusions: Defibrotide is a well tolerated agent that could be useful for the prophylaxis of VOD in patients treated with GO. Further studies are needed to confirm the effectiveness to prevent VOD.

**PO-015**

**EARLY MORTALITY RATE IN ACUTE MYELOID LEUKEMIA PRESENTING WITH HYPERLEUKOCYTOSIS**

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Acute myeloid leukemia (AML) presenting with hyperleukocytosis is generally considered of poor prognosis due to increased early death rate (within 7 days from the beginning of therapy) and a low response to initial chemotherapy. Early mortality reported in literature is 20–40% and it is particularly frequent when initial white blood cell count (WBC) is above 100×10⁹/L, due to intracerebral hemorrhage or respiratory distress related to leukostasis. Some studies have proposed leukapheresis to mechanically lower the number of leukemic cells in peripheral blood in order to prevent these fatal complications (Bunin and Pui 1985; Porcu et al. 1997). Between March 1995 and March 2004, 42 patients with newly diagnosed AML, median age 56 years (range 17–80 years), admitted to our institution with an initial WBC higher than 100×10⁹/L (median 157×10⁹/L, range 102–345×10⁹/L) were scheduled to receive chemotherapy without leukapheresis using hyperhydration, uricosuric agents and urine alkalinization. After induction therapy complete remission rate (CR) was achieved in 13 patients (30%). Early death occurred in 9 cases (21%), 7 due to intracerebral hemorrhage and 2 to respiratory distress. In 2 cases early death occurred before beginning chemotherapy. In the oth-
MULTIPARAMETRIC IMMUNOPHENOTYPING IN ADULT ACUTE LEUKEMIA: CORRELATION WITH KARYOTYPIC ABNORMALITIES

PO-016


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Immunophenotyping is an essential method for the diagnosis and classification of acute lymphoblastic leukemia (ALL) and is particularly important for the correct identification of minimally differentiated acute myeloid leukemia (AML). Furthermore, immunophenotyping has been reported to be able to detect aberrant phenotypes, to identify minimal residual disease and to correlate with structural chromosomal abnormalities such as t(15;17), t(8;21) and inv(16) in AML and t(9;22) and t(4;11) in ALL. We evaluated the correlation between immunophenotypic findings and karyotypic abnormalities in a large cohort of adult acute leukemia patients to assess whether immunophenotyping can suggest the presence of structural chromosomal abnormalities. In a group of 415 cases of adult acute leukemia (125 B-ALL, 290 AML); median age 47 yrs. (range 14–91), we analyzed the immunophenotypic findings, investigated with a large panel of monoclonal antibodies, together with the karyotypic abnormalities, detected by conventional cytogenetics and/or RT-PCR. According to the FAB criteria there were 14 M0, 26 M1, 87 M2, 72 M3, 5 M3v, 37 M4, 6 M4eo, 27 M5, 12 M6 and 4 M7 cases. Of 125 B-ALL cases 28 (22%) had t(9;22) had t(9;22) and 6 (5%) t(4;11). In the AML group t(15;17) was found in all M3 cases, while t(8;21) was observed in 7% (1 M0, 16 M2, 2 M4) and inv(16) in 3% (1 M2, 7 M4eo) of AML cases. In ALL with t(9;22) we observed a significantly higher expression of CD10, CD34 and CD13 (p=0.007, 0.014, 0.036, respectively) while n ALL with t(4;11) CD10 was absent but there was a high frequency of CD15 expression (p=0.001). The predictive value of immunophenotyping for t(9;22) was even more evident (p=0.0001) when antigen coexpression (CD10/CD13/CD34/CD34) was investigated. In M3 cases we found a lower expression of HLA-DR and CD34 (p<0.001) and a higher expression of CD2 (p=0.02); all but one variant M3 (80%) were CD2+ vs. only 16 of 72 (22%) classical M3. The t(8;21) cases demonstrated a significantly higher frequency of CD19 (p<0.001) and CD56, CD34, CD15.HLA-DR expression (p=0.05); in cases with inv(16) a high frequency of CD2 expression (p=0.003) was observed. Our results confirm the correlation reported between some structural chromosomal abnormalities and antigen expression; however, CD13 and CD15 expression in B-ALL does not seem to be confined to cases with t(9;22) and t(4;11), respectively. In AML cases, too, there seems to be a strong but not strict correlation between CD19 and CD2 expression with t(8;21) and inv(16), respectively, and lack of HLA-DR, CD34 and CD15 expression in cases with t(15;17). Our study confirms data in the literature and suggests that multiparametric and quantitative immunophenotyping contribute to classify distinct subgroups of acute leukemias.

A MAINTENANCE TREATMENT WITH 13-CIS RETINOIC ACID + DIHYDROXYLATED VITAMIN D3 + LOW DOSE CHEMOTHERAPY CAN PROLONG REMISSION DURATION AND SURVIVAL IN OLD /POOR RISK PATIENTS WITH AML OR MYELODYSPLASTIC SYNDROME

PO-017

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AML and high risk myelodysplastic syndrome (MDS) patients above the age of 60 have usually a dismal prognosis, in spite of aggressive chemotherapy treatment. Indeed, while complete remission (CR) rate is now around 60%, early relapses usually occur, with median CR duration and survival around 9–12 months (Sekeres MA et al.: Curr Opin Oncol 2002;14: 24–30; Pulsoni A et al.: Haematologica 2004;89:296–302). On the basis of our previous experience with MDS and AML patients unsuitable to intensive chemotherapy (Ferrero D et al.: Leuk Res 1996; 20: 867–76; Haematologica 2004;89:619–620), we adopted a combination of 13-cis retinoic acid (Roaccutan) 20–40 mg/day + 1,25(0H)2 vitamin D3 (Rocaltrol) 1 µg/day, associated to intermittent low dose chemotherapy, as a maintenance treatment to 20 AML and 8 MDS patients in CR after different schemes of intensive chemotherapy but at high risk of relapse. Nineteen patients had received a consolidation treatment while 9, for poor clinical conditions, proceeded...
directly to maintenance therapy. Low dose chemotherapy consisted, in the first 11 patients, of 6-thioguanine (Thioguanine) 40 mg/day×3/5 weeks. In the next 17 patients a 14 day course of ARA-C (Ara-cytin) 8 mg/m² s. c./2/day + 6-mercaptopurine (Purinethol) 50 mg/day was substituted for one 6-thioguanine course every 3 months. The whole treatment started after a median of 2.5 months (0.5-4) from CR achievement and was continued until relapse or 4 years of continuous CR. The median age of the 28 patients was 65.5 (29-73). All patients were ineligible to allogeneic BMT and shared one or more poor prognostic factors, including age >60 (21), therapy-related disease (4), previous relapse (2), resistance to first induction treatment (3), leukocytosis >20×10⁹/L (6). Cytogenetic analysis was successfully performed in 18 patients: 12 displayed a normal karyotype while unfavourable chromosome abnormalities were detected in 6. The treatment was well tolerated, without major toxicity. After a median follow up of 37 months (9-62), median CR duration and survival of the whole group of patients, as well as of patients above the age of 60, reached 22 (1-61+) and 23 (4-63+) months, respectively, with a 25% 4 year actuarial survival. Diagnosis (AML vs. MDS), type of induction therapy with/without consolidation treatment did not affect CR duration or survival. Inclusion of low dose ARA-C in the maintenance therapy was associated with a trend towards a longer CR duration that, however, did not reach a statistical significance. Conversely, the 12 patients with a normal karyotype displayed a significant longer CR and survival (35% and 40%, respectively, at 4 years; p=0.03 and 0.049) compared to those with abnormal or unknown karyotype (10% at 4 years). In conclusion, our maintenance therapy confirmed, at a longer follow up, the prolongation of CR and survival, compared to literature data, in a group of poor prognosis AML and MDS patients and is probably worthy to be tested in a larger casistic.

**PO-018**

**PERICENTRIC CHROMOSOME 8 INVERSION ASSOCIATED WITH THE 5’RUNX1/3’CBFA2T1 GENE IN ACUTE MYELOID LEUKEMIA CASES**

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**Introduction.** Translocation t(8;21)(q22;q22) is a common karyotypic abnormality detected in about 15% of acute myelocytic leukemia (AML) cases involving the CBFA2T1 (also known as ETO) and RUNX1 (also known as AML1) genes. Moreover, insertions generating the 5′RUNX1/3′CBFA2T1 gene have been described from 1 to 8.5% of AML cases. We report two AML cases with the 5′RUNX1/3′CBFA2T1 fusion gene on der(8) short arm, generated by a pericentric chromosome 8 inversion followed by a t(8;21) translocation. A detailed molecular cytogenetic characterization of breakpoints has been performed.

**Design and Methods.** The two cases were identified during screening of 82 AML patients bearing the RUNX1/CBFA2T1 rearrangement detected by RT-PCR. Both patients were diagnosed and tested by conventional cytogenetic analysis and fluorescence in situ hybridization (FISH). Results. FISH co-hybridization experiments with CBFA2T1 and RUNX1 probes revealed, in both cases, the presence of a single unexpected fusion signal on the 8p derivative chromosome in addition to single signals on normal 8 and 21 chromosomes. Moreover, faint CBFA2T1 and RUNX1 signals were observed on the long arm of der(8) and der(21) chromosomes, respectively. These data suggested that a pericentric chromosome 8 inversion involving CBFA2T1 gene occurred and that the chromosome 21 was rearranged with the 8p derivative chromosome. To define the breakpoint on the 8p derivative chromosome, we performed FISH experiments with appropriate probes located at chromosome 8 short arm. These studies revealed different breakpoint locations at 8p21.3 and 8p21.1. **Discussion.** FISH pattern observed in the two reported cases is caused by a t(8;21) translocation following a chromosome 8 pericentric inversion. To date, pericentric inversions accompanying t(8;21) translocation have never been reported. Our results confirm the critical role that the 5′RUNX1/3′CBFA2T1 fusion gene plays in leukemogenesis independently from its location. These data suggested that the mechanism leading to the 5′RUNX1/3′CBFA2T1 chimeric gene is heterogeneous, as it can be associated with different chromosomal rearrangements.

**PO-019**

**CLINICOBIOLGIC FEATURES IN 5 PATIENTS WITH ACUTE MYELOGENOUS LEUKEMIA AND A NOVEL TRANSLOCATION T(2;3)(P21;Q26)**


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Chromosome translocations involving the EVI1 gene at 3q26 were detected in 1–3% of de novo acute myelogenous leukemia (AML). In the majority of cases, the inv(3)(q21q26) or the equivalent t(3;3)(q21;q26) define the 3q21q26 syndrome, consisting of AML/MDS with normal or elevated platelet count and dismal prognosis; however other rare 3q26 translocations were described, the significance of which is not known. A novel balanced translocation, t(2;3)(p21;q26) was found in 5 patients with acute myelogenous leukemia, 4 of whom were drawn from an unselected series of more than 900 patients enrolled in the GIMEMA trials since 1999. These patients were characterized by molecular cytogenetic studies and by hematologic studies. The t(2;3)(p21;q26) was the stemline-defining anomaly in all cases; additional chromosome aberrations in two patients were +15 and +14. In 3 cases EVI1 splitting was detected by FISH; studies are in progress for the remaining two patients. EVI1 expression was tested by qPCR in 4 patients using normal BM cells as control, using primers able to discriminate between the EVI1 and MDS-EVI1 transcripts. An 8–10-fold EVI1 overexpression was detected in all patients, whereas MDS-EVI1 overexpression was not detected. Median age at presentation was 50 years (range 36–59); WBC count ranged between 2.9×10⁸ and 122.6 (median 6.2); Hb levels ranged between 8.3 gr/dl and 9.7 (median 8.7); the median platelet count was 104×10⁹ (range 54–171). Moderate splenomegaly was observed in 1 patient. The FAB diagnosis was AML-M4 in 2 cases, AML-M1, M2 and M5 in 1 case each. Multilineage dysplasia as defined by the WHO group was seen in 3 out of 4 cases. CD34⁺ was observed in 4 out of 5 cases, CD13 and/or CD33, and or MPO tested positive in all cases, lymphoid markers were not expressed, with the exception of CD7 positivity in 1 patient. Despite aggressive treatment including anthracycline plus cytarabine in conventional doses, with or without etoposide, complete remission was achieved in 1 patient only, who showed prolonged thrombocytopenia after induction. Remission was not achieved after salvage treatment, with the exception of a 4-month response in 1 patient following the FLAG regimen. Aploidentical allogeneic bone marrow transplantation achieved CR in 1 patient who subsequently died of infection; only partial short lasting response was seen in 1 patient submitted to unrelated-donor BMT. One patient is alive in first CR at 6 months; 4 patients died after 4–14 months. These findings suggest that the t(2;3)(p21;q26) is a recurrent primary chromosome aberration in de novo AML identifying a specific disease subset with the following distinctive features: i) EVI1 involvement and overexpression; ii) MDS features of the nonblast cell population; iii) severe clinical outcome due to primary chemoresistance.
The treatment of relapsed adult acute lymphoblastic leukemia (ALL) is frequently unsuccessful with current chemotherapy regimens and often there is an overexpression of Multidrug Resistance (MDR) related proteins. Liposomal encapsulation make daunorubicin less sensitive to the efflux effect of P-glycoprotein (PGP) and in vitro data indicate that liposomal-encapsulated daunorubicin (DaunoXome-DNX) is more toxic than daunorubicin (DNR) against ALL cell lines. In this study we assessed the in vivo and in vitro efficacy and toxicity of DNX and Cytarabine (Ara-C) as reinduction chemotherapy in 25 relapsed ALL patients (pts). The expression of MDR related proteins (PGP, MRP, LRP) was also analyzed. There were 12 males and 13 females, median age 35 yr (range 16-58), 6 ALL T and 19 ALL B, Ph+ 8/25 (32%), Bcr-Abl + 9/25 (36%). The leukemic cells expression of MDR and the DNR and DNX retention and induction of apoptosis were evaluated in all cases. Seventeen of 25 (68%) pts were at first relapse and eight (32%) at second or subsequent relapse. Nine (36%) pts relapsed after an allogeneic BMT. DNX was given at the dose of 80 mg/m2/day (days 1-3) in the first 11/25 pts (44%) and then at the dose of 100 mg/m2/day (days 1-3) in 14/25 (66%) of cases. In all pts Ara-C was administered at the dose of 2 g/m2 (days 1-5). Twenty pts (80%) achieved a complete remission (CR) and 2/25 (8%) entered a partial remission (PR) for an overall response (OR) rate of 88% (22/25) with a tolerable toxicity and without significant cardiotoxicity. At relapse 18/25 (72%) cases overexpressed at least one MDR related protein compared to 9/25 (36%) cases at diagnosis (p=0.01). The response rate was not affected by MDR overexpression and in vitro results showed an higher uptake and apoptotic cell death of DNX compared to DNR. Twelve pts subsequently underwent autologous bone marrow transplantation (11 MUD-BMT, 1 sib- lling BMT). The overall survival was 43% at 12 months. These data show the efficacy (OR rate 88% and CR rate 80%) of DNX plus Ara-C as reinduction therapy in very poor-risk ALL and also confirmed that DNX can overcome the MDR in vivo. The co-administra- tion of G-CSF could reduce the hematologic toxicity by improving granulocyte recovery. Moreover the high rate of remissions and good clinical tolerance in pre-treated pts also suggest a possible role of DNX in front line ALL chemotherapy regimens.

Although patients with acute lymphoblastic leukaemia (ALL) can achieve complete clinical remis- sion, many ultimately relapse. The relapse results from residual cancer cells that persist in patient below the limits of detection by standard techniques. Therefore, considerable effort has been directed at developing techniques that sensitively detect minimal residual disease (MRD). Molecular investigations of MRD levels and the dynamics of MRD in childhood ALL has proven superior to the other standard criteria (age, sex, and WBC) in distinguishing patients at high, intermediate and low risk of relapse. To date clinical implications of MRD in adult ALL patients have been less investigated. Measurement of MRD can be obtained by Real-time quantitative PCR (RQ-PCR) analysis of immunoglobulin and T-cell receptor gene rearrangements, breakpoint fusion regions of chromosome aberrations or fusion-gene transcripts. In this study we included 10 B-cells ALL patients (median age 33, range 18-54 years), recruited at ours institutions. Molecular studies were performed at diagnosis to identify a MRD-PCR target for each patient. In eight patients an unique IgH gene rearrangement was found and in two patients a BCR-ABL rearrangement (one p190 and one p210). The molecular rearrangement of the IgH genes was studied by sequencing on a ABI PRISM 310 Genetic Analyzer (Applied Biosystems- Big Dye Terminator Cycle Sequencing Kit). Patient specific oligonucleotides and probes were designed spanning the V-D-J junctional regions (PrimerExpress software). We performed RQ-PCR assays on an ABI PRISM 7900 platform with TaqMan probes and to determine the sensitivity and accuracy of patients specific MRD assay we per-
formed serial dilutions of known amounts of diagnostic DNA in normal DNA. These experiments demonstrate that a sensitivity higher than $10^{-4}$, $10^{-5}$ has been reached for all patients. We examined the reproducibility of the method by comparing results obtained from triplicate samples. We used a Relative Quantification method by comparing target and control genes cycle threshold ($\Delta\Delta Ct$). A single copy Telomerase gene TEL was selected as control gene. BCR-ABL fusion gene expression was analyzed using oligonucleotides, probes and control gene (Abelson) standardized in Europe Against Cancer (EAC) program protocol. Eight patients were evaluated for the outcome analysis (median follow up 12mo, range 7-40) the others two pts were actually excluded from evaluation because of a shorter than 3 months follow-up. Five pts showed a reduction of MRD level $>$3log after induction therapy, one achieved a 3 log reduction MRD only after consolidation therapy; all these pts are in complete remission at a mean follow-up of 18 months. The others two pts had a lower rate decrease therefore they were considered bad responders. One bad responder patient received an allogeneic bone marrow transplantation six months after diagnosis and achieved a $>$3log reduction after transplant, the other bad responder patient showed a persistent and increasing level of MRD during the follow-up and subsequently relapsed 21 months after the diagnosis. The present study involves only few patients and the follow up is relative short but it is clear that this method provides interpretable data that can be applied for risk assignment in adult ALL such as in paediatric setting.

**PO-022**

**EARLY DETECTION OF RESIDUAL DISEASE BY Q-RT-PCR OF THE HYBRID BCR/ABL TRANSCRIPT IS A POWERFUL PREDICTOR OF TREATMENT RESPONSE IN ADULT PHILADELPHIA-POSITIVE ACUTE LYMPHOBLASTIC LEUKEMIA**

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Ph+ acute lymphoblastic leukemia (ALL) is a subgroup of ALL with dismal prognosis and high risk of treatment failure. Despite high dose chemotherapy induces complete response (CHR) in 70-80% of Ph+ ALL patients, most of them experience early relapse. Imatinib has been shown to induce a CHR in about 60% of patients with relapsed or refractory Ph+ ALL. However median time to progression is only 2.5 months. Recent studies showed that childhood Ph+ ALL show a heterogeneous response to treatment and that response to prednisone and molecular response may predict remission duration and overall prognosis. In this prospective study we evaluated the level of residual disease in a series of 45 adult Ph+ ALL patients enrolled into the GIMEMA ALL0496 and LAL2000 protocols, to verify whether early detection of chemosensitivity may be used to predict the outcome and to identify patients at risk of early relapse. Both protocols included an induction period with high dose daunorubicin and a consolidation treatment according to the HAM protocol. Based on the availability of a HLA-identical siblings, allogeneic hemopoietic stem cell transplantation (SCT) was scheduled. The levels of residual BCR/ABL transcripts were assessed in bone marrow samples twice (after induction and after consolidation) by a Q-RT-PCR assay standardized from our group in the context of the European Study Group on MRD. Four patients resistant to induction and two with a clinical follow-up shorter than 6 months were excluded from evaluation. Thirty-nine patients were evaluable for the outcome analysis (median follow-up 14mo, range 6-48). Twenty-one patients had the P190 type of BCR/ABL junction, five the P210, and three cases had both types of transcripts. At diagnosis the mean level of BCR/ABL transcript, expressed as ratio to the GUS control gene, were 1.20 (range 0.23-10.30); no difference was found between patients expressing P190 and P210. Patients were operationally divided into two groups based on the Q-RT-PCR assay of bone marrow BCR/ABL mRNA levels: 27 patients with a level of residual disease below 0.01 ($>2$ logs of reduction) after induction therapy, and below 0.001 ($>3$ log of reduction) after consolidation therapy were considered good molecular responders (GMRs), while the remaining 12 cases who, despite the achievement of CHR, had a lower rate of decrease of residual disease at both time points, were considered poor molecular responders (PMRs). Clinical follow-up showed that early reduction of residual leukemic mass is an independent and powerful prognostic parameter. In the GMRs the actuarial estimated probability of relapse-free survival, disease free survival and overall survival at two years was 31%, 44%, and 44%, as compared to 0%, 0%, and 0% in the PMRs ($p = 0.0029, 0.001$ and 0.03, respectively), without regard to transplant procedure, which were per-
formed in half the patients in both groups (13/27 in GMRs and 6/12 in PMRs). At the end of study 13 GMR patients and only one PMR were alive. Furthermore, only in four cases, all belonging to the GMR group, salvage therapy, including Imatinib, resulted in a second remission. Our data indicate that, as documented in the children, also adult Ph+ALL comprises patients with heterogeneous sensitivity to treatment, and that early quantitative assessment of residual disease is a powerful prognostic parameter in this disease.

Funding: Supported by Associazione Italiana per la Ricerca sul Cancro (AIRC), and COFIN, Ministero dell'Istruzione e Ricerca Scientifica (MIUR), Regione Campania, Intas (Brussel), EurLeukemiaNet (Brussel), Biogem (Avellino).

**PO-023**

**INTEGRATED ANALYSIS OF GENE EXPRESSION AND SINGLE NUCLEOTIDE POLYMORPHISMS: IDENTIFICATION OF A DISTINCTIVE GENE EXPRESSION SIGNATURE ASSOCIATED WITH LOH ON CHROMOSOME 9P IN ADULT ALL**

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**Background.** In acute lymphocytic leukemia (ALL), previous studies have demonstrated that specific genetic rearrangements are associated with distinctive gene expression signatures defined by high-density oligonucleotide microarrays. Characteristic expression signatures associated with ALL1/AF4, E2A/PBX1, BCR/ABL and TEL/AML1 rearrangements have provided insights into the mechanisms of malignant transformation and are also likely to improve the identification and classification of leukemia cells with these abnormalities. Nonetheless, many cases of ALL do not carry known molecular abnormalities and do not exhibit unique gene expression profiles. In these cases, the mechanisms of malignant transformation remain unknown. Aims. Our goal was to develop an integrated genetic approach to discover new mechanisms of malignant transformation in ALL and to define the gene expression signatures associated with these genetic abnormalities. Methods. Gene expression profiles were determined in leukemia cells from 95 adult patients with B-lineage ALL using Affymetrix U95Av2 GeneChips. Within this group, high density SNP analysis was performed on 18 samples of normal hematopoietic cells and tumor cells from the same patients using Affymetrix GeneChip Mapping 10K Arrays. Patients were enrolled in the Italian protocol GIMEMA 0496. Results. Paired SNP analysis of normal and ALL DNA revealed the presence of LOH in a portion of chromosome 9p (9p13.3 to 9p24.3) in 4 of 16 cases (25%). This deletion was not detected using conventional cytogenetic methods. FISH analysis confirmed the presence of a homozygous deletion at 9p21 in 2 of these samples, whereas the 2 remainder cases showed a normal copy number, suggesting that in these 2 patients a deletion followed by duplication occurred. Analysis of gene expression based exclusively on genes located in this area identified two genes differentially expressed: ADFP was found more highly expressed, whereas a transcript whose function is unknown was found expressed at low levels. Analysis of gene expression in these cases revealed increased expression of a set of 20 genes and reduced expression of a set of 18 genes. Using this gene set, we examined a larger series of 40 adult ALL cases without previously defined molecular rearrangements. This analysis identified 1 additional case with a similar pattern of gene expression, suggesting that also this sample may have a LOH in 9p region. Summary. These results show that SNP analysis is a powerful tool to identify genetic abnormalities that are not detected by conventional cytogenetic techniques. Furthermore, these results demonstrate that integrated analysis of gene expression combined with SNP-based comparison of leukemia and normal cells can identify previously unknown groups of tumors with distinct genetic profiles. Further studies can now be undertaken to better define the genetic mechanisms of malignant transformation in these leukemias.

**PO-024**

**TUMOR LYMPH SYNDROME IN ACUTE MATURE B-LYMPHOBLASTIC LEUKEMIA (L3 ACCORDING FAB CLASSIFICATION) PATIENT**

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Tumor lysis syndrome (TLS) is a life-threatening metabolic complication that occurs in malignancies with large tumor burden and highly proliferative cells, including lymphoma and leukemia. The lysis of tumour cells and the subsequent release of the intracellular contents leads to hyperkalemia, hyperphosphatemia, hyperuricemia, hypocalcemia, increased lactic dehydrogenase (LDH), activation of disseminated intravascular coagulation, and often renal
function impairment. These complications may lead to acute renal failure and dialysis and therefore have a negative impact on the outcomes of patients with malignancies that otherwise are potentially curable. We described a case of tumor lysis syndrome in acute B-lymphoblastic leukemia patient treated with administration of rasburicase, a recombinant urate oxidase that converts uric acid into the soluble compound allantoin. A 68 year old woman was referred to our emergency department for hyperleucocytosis, anemia, mild decrease in platelet number and acute renal failure. At the anamnesis the patient revealed five days of persistent fever (38.5°C) with mild dispnea, treated with a wide spectrum antibiotic (oral amoxicillin with clavulanic acid) and two doses of 20 mg of methilprednisolon in 24 hours. The patient was admitted in our hematology department and a central venous line was positioned in the suclavian vein. An i. v. therapy with 2000 ml/day per square meter of 0,9% saline solution associated with furosemide 1,5 mg/Kg i. v./day was started. In addiction, sodium bicarbonate infusion was administered and stopped when a urine pH > of 7,5 was reached. In order to obtain a rapid reduction of uricemia blood levels and improve the renal failure, rasburicase at the dose of 0,20 mg/Kg/day in 50 ml of 0,9% saline solution was administered in 30 minutes for seven days. At the same time, a complete hematological evaluation with bone marrow aspirate for morphologic, immunologic (TdT-, HLA-DR+, CD10-, CD19+, CD20+, CD24+, Sig+, µCyt.) and cytogenetic analysis [(t(8;14)(q24;q32)] was performed and the diagnosis of mature acute B-lymphoblastic leukemia (L3 according FAB classification) was done. We also performed a complete cardiac evaluation. After 12 hours form hospital admission, because of the rising leukocytes number levels, and the progressive normalization of renal function a chemotherapy treatment according B-NHL 83 protocol (Hoelzer et al.) was started. Treatment described was efficient in preventing the patient from dialysis treatment and from delay in chemotherapy treatment. In particular, rasburicase showed a great efficiency in reducing blood uricemia level within few hours after the first dose. It is efficient within 12 hours after the treatment, for renal function in patients with blood uricemia level. TLS is poor complicate in treatment of high growth fraction. Classical prophylaxis of tumor lysis syndrome consists of hydration, alkalinization, and use of the xanthine oxidase inhibitor allopurinol. The limitations of allopurinol include slow onset of action, insufficient efficacy in many high risk patients and the ability to participate in significant drug-drug interactions with common chemotherapeutic agents. The use of allopurinol leads to the accumulation of xanthine, which may crystalize and precipitate in the renal tubules. Urate oxidase acts by converting uric acid into allantoin, which is 5–10 times more soluble than uric acid and therefore is rapidly excreted by the kidneys.

**PO-025**

**MONITORING MRD IN PH POSITIVE ACUTE LYMPHOBLASTIC LEUKEMIA BY THREE DIFFERENT MARKERS**

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Acute lymphoblastic leukemia (ALL) is a clonal disorder of hematopoietic stem cells resulting in proliferation and expansion of leukemic cells. Progress in our understanding of molecular mechanisms has made it possible to increasingly recognize the role of molecular abnormalities in the leukemogenic process. We have studied the expression of three bio-molecular markers involved in the pathogenesis and progression of ALL. The Philadelphia chromosome (Ph) and its molecular product, the Bcr–Abl fusion protein p190, are the most frequently detected cytogenetic-molecular abnormalities in adults ALL patients, resulting in a worse prognosis. Another useful tool to investigate every lymphoproliferative disorders is the IgH gene rearrangement which has been shown to be an easily detectable marker of minimal residual disease (MRD). Recent studies have revealed that in acute leukemias there is an overexpression of Wilm's tumor 1 gene (WT1). Therefore this phenomenon might be exploited as a marker to establish the presence, the persistence or the reappearance of leukemic hematopoiesis. We have studied five Ph positive, CD10 positive ALL patients. Three out of them have been treated with Fludarabine-containing regimen, the other have received a combination of vincristine (VCR), cyclophosphamide (CTX), daunomicin (DNM), and steroids as induction therapy. Two patients who had a matched sibling donor underwent allogenic bone marrow transplantation in cytogenetic complete response, both of them have died, one for recurrent disease, the other for transplant related toxicity. Three patients who were not eligible for high dose therapy, were maintained with 600 mg/day imatinib-mesylate therapy for 45 days, followed by daily mercaptopurine and weekly metotrexate for 45 days, and a re-induction VCR CTX, DNM and steroids. All these patients are still alive in good clinical conditions and in cytogenetic complete response. We have studied the expression of BCR-ABL qualitative, WT1 quantitative and IgH gene rearrangement in these five
Our data shows the feasibility and the usefulness of monitoring MRD by three different markers. In our hand the most predictive index is IgH monoclonal rearrangement. The prognostic value of the expression of WT1 gene is still under investigation. After the advent of Imatinib a new scenario may be represented. The patogenetic step of leukemogenesis can be studied and the above mentioned neoplastic markers might acquire a different prognostic value. The lineage specific extrinsic neoplastic marker, that is IgH rearrangement, may identify a clonal instable population that has not yet acquired the intrinsec marker (the p190 transcript). The overexpression of the WT1 gene may identify the clonal instability of this leukemic clone. So the presence of p190 transcript represents the last patogenetic event and is a strong index of impending relapse. We think that the identification of MRD at a precox patogenetic step is the goal of the treatment of Ph positive ALL. WT1 expression may be the simplest method to stratify the patients in different prognostic and therapeutic subgroups.

**PO-026**

**P-ERK1/2 and P21CIP-1/WAF1 in acute lymphoblastic leukemia cells: effects of the MEK inhibitor PD98059**

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The mitogen activated protein kinase (MAPK) pathway links cellular signals (proliferation, differentiation and apoptosis) from the cell surface to the cytoplasmic/nuclear events. Extracellular signal-regulated kinase-1/2 (ERK) are serine/threonine kinases involved in the MAPK pathway. Prolonged MAPK activation may increase one of the cell cycle negative regulators, the p21CIP1/WAF1 (p21) expression, resulting in cell cycle arrest and cytoprotective effects against chemotherapeutic agents. The aim of our study was to evaluate the role of phospho-ERK (p-ERK) and p21 in leukemia cell lines and in primary blasts from 118 adult acute lymphoblastic leukemia (ALL). Expression of p-ERK and p21 were evaluated by flow cytometry using a specific monoclonal antibody for p-ERK1/2 (clone E10) and p21CIP-1/WAF1. Phospho-protein expression was analyzed using the Kolmogorov-Smirnov statistic test (D-value). Results were confirmed by Western blot analysis. Human K562 and RPMI8866 cells express p-ERK (D-value=0.47 and 0.38, respectively), as well as normal PMA-activated PBL (D-value=0.37), whereas resting PBL and normal CD34+ cells exhibited minimal levels of p-ERK (D-value=0.04 and 0, respectively). Only the RPMI8866 cells expressed p21 (D-value=0.32), whereas the K562 did not; resting PBL exhibited low p21 levels (D-value=0.18). Expression of p-ERK (D-value >0.10) was found in 38/118 adult ALL cases (32.2%), ranging between 0 and 0.72, while 44.9% of cases (53/118) showed p21 levels higher than 0.15.Both proteins were significantly associated with higher WBC values (>20x10^9/L): p=0.015 and p=0.04 for p-ERK1/2 and p21, respectively. No correlation was found between other clinical characteristics (age, sex, leukemia phenotype). A proportion of samples was in vitro exposed to the MEK inhibitor PD98059 (25 mM) which induced a significant (p=0.014) downregulation of the p-ERK expression (from a D-value of 0.09±0.11 at time 0 to 0.03±0.06 after 24h). In contrast, an increased expression of p21 (D-value 0.15±0.07 and 0.32±0.29, respectively; p=0.015) was noticed following treatment with the MEK inhibitor. In summary, our study shows that both p-ERK and p21 are expressed in a significant proportion of adult ALL samples and both p-ERK appear to be associated with higher WBC counts. The MEK inhibitor
PD98059 was able in vitro to downregulate expression of p-ERK1/2 in samples from ALL patients. The importance of these results in terms of chemosensitization of ALL cells is under evaluation.

PO-027
EXPRESSION OF BCRP IN ADULT ACUTE LYMPHOBLASTIC LEUKEMIA: ROLE AND CORRELATIONS WITH OTHER MDR-RELATED PROTEINS
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The transporter breast cancer resistance protein (BCRP) was recently described as an additional multidrug resistance protein in solid tumors (breast, gastric and colon cancer) and in acute myeloid leukemia (AML). However, the role of BCRP in acute lymphoblastic leukemia (ALL) remains to be established. The aim of our study was to evaluate in samples from adult ALL patients the frequency of BCRP expression and its correlation with other MDR-related proteins: the P-glycoprotein 170 (P-gp170), the multidrug resistance-associated protein (MRP1) and the lung resistance protein (LRP). Human leukemic cell lines and primary samples from 110 untreated ALL patients were evaluated for BCRP, P-gp170, MRP1 and LRP expression by flow cytometric techniques. BCRP protein expression was analyzed by the monoclonal antibody BXP-34 and the analysis was performed by the Kolmogorov-Smirnov (KS) statistic test (D-value).

Detection of BCRP in the cell lines MCF7 pcDNA3 and MDA231 pcDNA3 showed lower protein levels (D-value= 0.12±0.11 and 0.09±0.06, respectively), whereas the cell line MCF7 pcDNA3 clone8 and MDA231 pcDNA3 clone 23 expressed BCRP at higher levels (D-value= 0.44±0.21 and 0.33±0.11, respectively). Analysis of ALL primary samples showed a BCRP expression (D-value >0.15) in 69/110 (62.7%) cases, with a mean value of 0.32±0.19 (range 0.00-0.87, median 0.33) in the overall population. BCRP expression resulted higher (0.34 ± 0.03) in samples from patients with white blood cell (WBC) counts >100×10⁹/L compared to a lower value (0.26±0.13) in patients with WBC less then 100×10⁹/L (p=0.06). No significant difference was found between BCRP expression and clinical characteristics. The analysis was then extended to the other MDR-related proteins: P-gp170 expression was detected in 21.7% (D-value/0.05) of cases, while MRP1 and LRP (D-value / 0.20) were found in 48.8% (mean 0.21±0.30, ranging 0.00–0.93) and 37% (mean 0.23±0.27, ranging 0.00–0.92) of cases, respectively. The majority of cases showing absence of MRP1 expression resulted negative for LRP (50.4%; p=0.001). Among the 110 samples, 72 of them were analyzed simultaneously for the expression of BCRP and MRP1 which resulted significantly correlated (R= 0.44; p= 0.0001): 45.8% of samples were negative for both proteins, while 13.9% expressed both BCRP and MRP1 proteins. In fact, MRP1 negative samples showed lower BCRP levels (m= 0.30, range 0-0.60) compared to MRP1 positive cases (m= 0.38, range 0-0.87) (p=0.017). The BCRP expression was not correlated with the P-gp170 and LRP expression. In summary, our study shows that BCRP, MRP1 and LRP are expressed in a significant proportion of adult ALL samples. BCRP expression is associated with a higher WBC count and higher MRP1 levels. MRP1 and LRP were also reciprocally associated. The role of the simultaneous expression of all these proteins in the chemoresistance of adult ALL patients is under evaluation.

PO-028
Not published

PO-029
CAN CONSTITUTIONAL ABNORMALITIES OF 11q23, NOT INVOLVING MLL, BE INVOLVED IN PATHOGENESIS OF CHILDHOOD ACUTE LEUKEMIA? A CASE REPORT ABOUT A CONSTITUTIONAL TRANSLOCATION T(11;13)(q23;q34) IN A CHILD WITH ACUTE LYMPHOBLASTIC LEUKEMIA
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11q23 abnormalities are often seen in childhood acute lymphoblastic leukemia, being characterized by a high heterogeneity of the partner chromosomal bands (at least 80 different loci) and in most cases disrupt the MLL gene which is thought to be crucial for leukemogenesis. We report about a 2.5-year-old child who was diagnosed B-acute lymphoblastic leukemia in June 2001. Flow cytometric analysis revealed a Common phenotype (CD19+·CD10+·CD34+·CD22+·cmyu-) with a very dim expression of myeloid antigens CD13 and CD33. Cytogenetic analysis showed the presence of 11/11 metaphases carrying a reciprocal translocation between the long arm of chromosome 11 (band q23) and that of chromosome 13 (band q34), resulting in a t(11;13)(q23;q34) translocation. This finding was initially interpreted as...
leukemia-related and thus coherent with literature data about 11q23 alterations in childhood leukemia, except for the partner chromosomal band 13q34 which resulted rarely reported. The patient was then followed-up with morphology, cytogenetics, immunophenotype at each step of therapy. Surprisingly, after induction, consolidation and even during maintenance, clinical and laboratory findings were consistent with complete hematologic remission as assessed by morphology and immunophenotype, while cytogenetics always showed all metaphases positive for t(11;13). Remission lasted until November 2003, when overt hematological relapse occurred exhibiting phenotypic switches, mainly a decreased expression of CD34 and an increased expression of CD33 myeloid antigen. Nevertheless, karyotype did not change and again all metaphases were t(11;13) positive. We thus postulated that the patient carried a constitutional rather than a leukemia-related translocation. The analysis of peripheral blood T-lymphocytes stimulated with PHA resulted strongly consistent with this hypothesis, as they carried the same translocation. Obviously, the translocation could have been occurred in a totipotent hematopoietic stem cell rather than in a somatic cell during embryonic development: a skin biopsy could confirm the last hypothesis, but to date it hasn’t yet been investigated. However, it seems to be an acquired rather than an inherited translocation, as the t(11;13) was not found in parents. Analysis of MLL rearrangement was performed by double-color FISH in bone marrow and peripheral blood of the patient, but it showed no MLL disruption, as the breakpoint in 11q23 was located outside and centromeric to MLL (350 kb distant from MLL bcr). Because of the availability of an unrelated HLA-matched female donor, the patient underwent allogeneic bone marrow transplantation in April 2004. At day +30 after transplantation, cytogenetics showed the disappearance of t(11;13) along with the presence of a 46,XX karyotype, thus documenting a prompt engraftment. In conclusion, if data will be consistent with this hypothesis, we could have probably found a novel candidate gene mapping in 11q23 such as RCK, LARGE, etc., known to be implicated in leukemogenesis, could be involved. If this is the case, we can speculate that this congenital 11q23 aberration could be responsible for the initiation of the leukemic process at an early stage of development and in turn this assumption provide new insights into the molecular pathogenesis of childhood acute leukemia.
(GADD153), prothymosin α (ProT-α; PTMA) and high mobility group protein (HMG-I). On the other hand, in the same group, we found a down regulation of retinoic acid receptor β (RXR-β; RXRB), DNA topoisomerase I (TOP1), interleukin-2 receptor α subunit, interleukin-6, protein kinase MLK-3, rap1 GTPase activating protein 1 (RAP1GAP), metalloproteinase inhibitor 1 precursor (TIMP1), RCL growth-related c-myc-responsive gene, hepatocyte growth factor-like protein. Summary/Conclusions: ER and ENR MDS patients present a different pattern of gene expression. These results may provide the basis for early testing of the genes differentially expressed in ER and ENR patients in order to elucidate their role in the prognostication of rhEPO response in MDS patients with low endogenous EPO levels.

PO-031
AN ALTERED LECAM1/ICAM1 RATIO ON CD34+ BLAST CELLS PREDICTS LEUKEMIC PROGRESSION IN MDS PATIENTS

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Interaction of adhesion molecules (AM) with bone marrow (BM) microenvironment regulates hematopoietic progenitor growth and survival. Consequently, the abnormal growth and proliferation observed in MDS/secondary acute myeloid leukemia (sAML) could be related to defective adhesive properties within the stem cell compartment. In this view we compared, in a three colour flow cytometric assay, the expression of β1-β2 integrins, Lecam1, CD44 and ICAM1 on CD34+ progenitor cells from BM of 66 patients (pts) affected by MDS (34 RA, 2 RARS, 23 RAEB, 7 RAEBt) or sAML (18) and 17 healthy donors (HD). The expression of AM was measured as percentage, ratio of mean fluorescent intensity (rMFI) and AM index (AMI = product of rMFI and percent positive cells). In the MDS/sAML group, a lower Lecam1 AMI was observed (p<0.001), whereas ICAM1 AMI was higher (p=0.024) as compared to HD. Given this reciprocal expression we designed a Lecam1/ICAM1 AMI ratio to distinguish HD from MDS/sAML CD34+ cells: a greater Lecam1/ICAM1 AMI ratio for HD as compared to MDS/sAML was observed (p<0.001). Furthermore, Lecam1/ICAM1 AMI ratio showed a strong inverse correlation with BM blast infiltration (r -0.53, p<0.001). This phenomenon was due both to the progressive ICAM1 AMI increase (r. 43, p=0.000) and to the reciprocal Lecam1 AMI decrease (r -0.30, p=0.011) occurring in pts with a high BM blast infiltration. In 2 pts with a ratio of 4.21 and 2.57, respectively, the leukemic progression was associated to a down regulation of Lecam1 and up regulation of ICAM1 so that their ratio reverted to 0.173 and 0.179, respectively. Two patients, undergoing intensive chemotherapy, had thei negative ratio reverted to normal positive values after a chemotherapy induced remission. In univariate analysis, the actuarial risk of disease progression was higher for MDS pts with a baseline ratio <1 (64% vs. 11% at 2 year, p=0.002), this significance being confirmed in multivariate analysis. In conclusion: 1) Lecam1 is defective in the stem cell compartment of MDS, whereas ICAM1 is overexpressed; 2) a baseline ratio <1 significantly predicts progression to overt leukemia; 3) after chemotherapy, the correction of the ratio may indicate the quality of remission.

PO-032
FLOW CYTOMETRIC ANALYSIS OF ERYTHROID AND MYELOID DYSPLASIA IN PATIENTS WITH MYELODYSPLASTIC SYNDROME

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Recent studies suggest that flow cytometry immunophenotyping might provide useful information in the management of myelodysplastic syndromes (MDS) patients. However, its role in the diagnosis of these disorders is still debated. The purpose of this study was to develop a flow cytometric approach to the evaluation of marrow dysplasia in MDS. We analyzed 103 consecutive MDS patients and 46 control subjects, including 27 pathologic controls and 19 healthy bone marrow donors. Discriminant function analyses were carried out to identify erythroid and myeloid dysplasia, with the aim of differentiating MDS and controls, and of classifying MDS into WHO subgroups. The erythroid and myeloid functions were prospectively evaluated on a series of 41 consecutive unselected patients (36 MDS, 5 pathologic controls). Erythroid and myeloid dysplasias were significantly higher in MDS than in controls (p<0.001), positively correlating with the degree of morphological erythroid dysplasia (r=0.53, p<0.001). The quantitative analysis of the GlyA/CD7 pattern showed a lower expression of CD71 on erythroblasts in MDS than in healthy subjects (p=0.02). In the myeloid compartment, a lower percentage of CD10+ and an increase in CD56+ granulocytes were noticed in MDS in comparison with controls (p<0.001). The quantitative analysis of
CD33/CD16, CD13/CD16, CD45/CD16, CD11b/CD16 patterns showed an increased proportion of immature cells with a reduction of mature granulocytes in MDS patients: calculated ratios of immature on mature cells resulted significantly higher in MDS than in controls (p values 0.02-0.001), and positively correlated with the degree of morphological myeloid dysplasia (r values 0.30-0.67, p<0.002). A significantly higher percentage of CD34+ cells was found in MDS than in controls (p=0.006), with a higher CD34+CD33+ to CD34+CD33- cell ratio (p<0.001). A erythroid classification function based on CD34+ cells (%), CD34+CD33+/CD34+CD33− ratio, erythroblasts (%) and GlyA/CD71 co-expression, and a myeloid function based on CD34+ cells (%), CD34+CD33+/CD34+CD33- ratio, CD10 and CD56 expression on myeloid cells, and CD33/CD16 immature to mature cell ratio were defined (Table 1), and combined with the immunophenotypic blast count.

The PRND gene, located on human chromosome 20pter-p12, 27 Kb downstream from PRNP, the prion protein gene, encodes for the Doppel protein (Dpl), that has many biochemical and structural properties in common with the prion protein. Both prion protein and Dpl are associated with neurodegenerative disease by elusive mechanisms. Dpl is about 179 amino acid residues long, it is characterized by an α-helical conformation, and it is presented on the cell surface by a glycosylphosphatidylinositol anchor. Whereas prion protein is ubiquitous, Dpl is expressed only during embryogenesis and in adult testis and does not seem to be required for prion disease progression, but appears to have an essential function in male spermatogenesis. Since preliminary studies have demonstrated a significant level of Dpl expression in several types of cancers of different histologic origin, we investigated the Dpl distribution in leukemic cell lines and in bone marrow cells from patients with acute myeloid leukemia (AML) and myelodysplastic syndrome (MDS), in order to evaluate its possible overexpression in these disorders. Molecular and immunocytochemical studies were carried out on the human leukemic lines HL-60 and K562 and on bone marrow samples from 16 normal controls, from 34 patients with AML, 25 de novo and 9 secondary, and from 59 patients with MDS (22 RA, 8 RARS, 13 RAEB, 7 RAEB-t and 9 CMML), not previously treated. A goat polyclonal antibody raised against a peptide mapping near the aminoterminus of Dpl of human origin (Santa Cruz Biotechnology, Inc.) was used for immunocytochemistry and Western blotting. Whereas normal samples were negative or showed very weak expression in rare immature myeloid cells, Dpl was detected in both cell lines and in most AML and MDS cases, with median percentages of positive blasts respectively of 14% (range 0-38%) and 16%.

### Table 1. Erythroid and myeloid classification functions*

<table>
<thead>
<tr>
<th>Cytometric variables</th>
<th>Erythroid function coefficients</th>
<th>Myeloid function coefficients</th>
</tr>
</thead>
<tbody>
<tr>
<td>CD34+</td>
<td>3.3161</td>
<td>9.1799</td>
</tr>
<tr>
<td>CD34+CD33+/CD34+CD33− ratio</td>
<td>7.0227</td>
<td>4.4653</td>
</tr>
<tr>
<td>Erythroblasts</td>
<td>12.4984</td>
<td>-</td>
</tr>
<tr>
<td>GlyA/CD71 erythroblasts</td>
<td>0.2115</td>
<td>-</td>
</tr>
<tr>
<td>CD10+ granulocytes</td>
<td>-</td>
<td>4.9664</td>
</tr>
<tr>
<td>CD56+ granulocytes</td>
<td>-</td>
<td>3.0147</td>
</tr>
<tr>
<td>CD10/CD56 immature to mature cells</td>
<td>10.8109</td>
<td>-</td>
</tr>
<tr>
<td>Constant</td>
<td>-19.0721</td>
<td>-17.4053</td>
</tr>
</tbody>
</table>

*The following variables were employed: X1 = log(CD34+), X2 = log(CD34+CD33+)/CD34+CD33− ratio, X3 = log(erythroblasts), X4 = log(GlyA+CD71+ erythroblasts), X5 = log(CD10+ granulocytes), X6 = log(CD56+ granulocytes), X7 = log(CD16/CD33 immature to mature cells).

The following classification functions were evaluated:

- Erythroid function: YE = 3.3161*X1 + 7.0227*X2 + 12.4856*X3 + 0.2125*X4 - 19.0721;

The use of these functions correctly classified 93.5% of MDS, with correspondence between cytometric and morphological diagnosis in 81% of patients. In 20 patients, who showed indeterminate marrow morphology at the diagnosis, the use of the classification functions allowed to a diagnosis of MDS in 12/20 cases. Strong positive correlations were found between the value of the discriminant functions and both the degree of morphological dysplasia (r=0.61, p<0.001; r=0.79, p<0.001), and IPSS (r=0.52, p<0.001; r=0.58, p<0.001). Finally, the functions were prospectively tested on a series of 41 consecutive unselected patients (36 MDS, 5 pathologic controls), achieving a correct classification in 36 out of 41 cases (87.8%). The immunophenotypic analysis of hematopoietic compartments provides an accurate evaluation of erythroid and myeloid dysplasia in MDS patients. A cost-effective panel of 8 monoclonal antibodies provides an accurate classification in most cases with indeterminate morphology, and might be an effective tool in the diagnostic workup of MDS patients.

**PO-033**

**OVEREXPRESSION OF THE PRION-LIKE PROTEIN DOPPEL IN ACUTE MYELOID LEUKEMIAS AND MYELODYSPLASTIC SYNDROMES**

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The use of these functions correctly classified 93.5% of MDS, with correspondence between cytometric and morphological diagnosis in 81% of patients. In 20 patients, who showed indeterminate marrow morphology at the diagnosis, the use of the classification functions allowed to a diagnosis of MDS in 12/20 cases. Strong positive correlations were found between the value of the discriminant functions and both the degree of morphological dysplasia (r=0.61, p<0.001; r=0.79, p<0.001), and IPSS (r=0.52, p<0.001; r=0.58, p<0.001). Finally, the functions were prospectively tested on a series of 41 consecutive unselected patients (36 MDS, 5 pathologic controls), achieving a correct classification in 36 out of 41 cases (87.8%). The immunophenotypic analysis of hematopoietic compartments provides an accurate evaluation of erythroid and myeloid dysplasia in MDS patients. A cost-effective panel of 8 monoclonal antibodies provides an accurate classification in most cases with indeterminate morphology, and might be an effective tool in the diagnostic workup of MDS patients.
which explored the potential therapeutic effects of we report the final results of a pilot clinical trial therefore, a synergistic effect could be possible. Here levels. This suggests that the mechanisms of action thalidomide may induce erythroid responses in some probability of responding to r-EPO. On the other hand, and reduced levels of endogenous EPO have the best transfusion-dependent patients with high serum EPO thalidomide, originally planned to confirm the possi- ders did not predict disease progression in MDS. In AML PRND expression was unrelated to the morpho- logical subtype and there was no correlation between Dpl levels and clinical or laboratory findings such as age, leukocyte count or karyotype. In conclusion, for the first time the expression of PRND has been demonstrated in human bone marrow cells. The molecular mechanism responsible for its overexpression in transformed cells is unknown; however, the differ- ential expression of the Dpl protein in AML and MDS versus healthy subjects makes it a possible leukemia associated antigen with a potential attractive role as target for immunotherapy; moreover, Dpl could be used to quantitate minimal residual disease after treatment. On the other hand, PRND expression in HL-60 and K562 cells may provide a model to study gene regulation and protein function.

PO-034
COMBINATION OF ERYTHROPOIETIN AND THALIDOMIDE: NO EVIDENCE OF A SYNERGISTIC EFFECT ON ANEMIA OF PATIENTS WITH MYELODYSPLASTIC SYNDROMES
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Previous studies have indicated that both recom- binant erythropoietin (r-EPO) and thalidomide may be effective in improving anemia of some patients with myelodysplastic syndromes (MDS). Characteristical- ly, subjects with no or very low need of transfusions and reduced levels of endogenous EPO have the highest probability of responding to r-EPO. On the other hand, thalidomide may induce erythroid responses in some transfusion-dependent patients with high serum EPO levels. This suggests that the mechanisms of action of these two drugs are probably different and that, therefore, a synergistic effect could be possible. Here we report the final results of a pilot clinical trial which explored the potential therapeutic effects of the combination of r-EPO and thalidomide in heavy- anemic MDS patients. Thirty transfusion-depen- dent patients (median Hb level 7.1 g/dl, range 5.3-8.5) with low-to-intermediate-1 risk MDS according to IPSS (18 males and 12 females, mean age 62.5 years, range 41-81), previously unresponsive to r-EPO (n. 15) or thalidomide (n. 15) employed as single agents, received r-EPO at the dose of 40.000/U s. c., once-weekly, in combination with thalidomide (100 mg/d p. o. for one week, to test tolerance, and then 200 mg/d) for 8-12 weeks. According to WHO clas- sification, there were 10 refractory cytopenias with trilineage dysplasia (RCMD), 13 refractory anemias (RA), 4 RA with ring sideroblasts (RARS)and 3 RA with excess of blasts type I (RAEB-1). Twenty-three patients did not evidence any erythroid response (IWG criteria) or did not tolerate thalidomide and stopped the treatment. Four patients within the group of those previously unresponsive to r-EPO alone achieved a hematological erythroid improve- ment (HI-E, 3 major, 1 minor) under combined ther- apy. In order to verify the real synergy of the associa- tion or the simple efficacy of the new drug adjunc- ted, after 12 weeks these responders continued with thalidomide alone. When r-EPO was withdrawn, all of them maintained their response, thus suggesting that only thalidomide was effective in these patients. Three of MDS previously unresponsive to thalidomide alone achieved a HI-E (1 major, 2 minor) after the adjunct of r-EPO. Again, when thalidomide was with- drawn, these patients maintained their response, thus suggesting the efficacy of r-EPO, but not that of the association, in these subjects. Since none of these patients lacked his response when re-allocated to the new single-drug arm, a further step with r-EPO plus thalidomide, originally planned to confirm the possibility of a synergistic effect in patients lacking their response after discontinuation of the combined therapy, was not necessary. Our data do not support the hypothesis of a synergistic effect for the association of r-EPO and thalidomide in MDS. It seems instead that two populations of patients can be identified, according to their sensitivity to r-EPO or, alterna- tively, to thalidomide.

PO-035
IN PATIENTS WITH MYELODYSPLASTIC SYNDROMES RESPONSE TO RHUEPO AND G-CSF TREATMENT IS RELATED TO AN INCREASE OF CYTOGENETICALLY NORMAL CD34+ CELLS
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In myelodysplastic syndromes (MDS) the use of recombinant human Epo (rHuEpo) has been extensively investigated with the aim to increase hemoglobin levels and to reduce transfusion requirement. To this extent, we recently demonstrated that response to rHuEpo might be related to the proliferation of karyotypically normal erythroid precursors possibly representing residual normal erythroid elements. Some clinical studies have shown that treatment with rHuEpo in combination with granulocyte colony-stimulating factor (G-CSF) may synergistically improve the rate of response with many long-lasting responses. Biological studies have demonstrated that G-CSF acts by inhibiting spontaneous cytotoxicity and apoptosis of MDS hematopoietic progenitors. However, it remains unclear whether, in vivo, the response to rHuEpo and G-CSF treatment is also related to the proliferation of residual cytogenetically normal CD34+ progenitor cells which are still present in the bone marrow of MDS patients. In the present work, by FISH analysis, we have therefore investigated, in bone marrow of MDS patients. In the present work, we analyzed the expression levels of MBN in 57 BM and 42 peripheral blood (PB) samples from 88 MDS patients 44 RA, 32 RAEB and 12 secondary-AML. As control, we analyzed the MBN expression in 15 BM and 40 PB samples from healthy volunteers. The expression level of MBN was established using quantitative RealTime PCR based on a specific set of primers and probe (Assays-on-Demand, gene expression products, Applied Byosystems). The values obtained were normalized using ABL as housekeeping gene and the final results were expressed using the 2^(-ΔΔCt) method. The numerical values are expressed as 2^-ΔΔCt. We found that MBN gene is significantly overexpressed in BM samples from AML patients. The mean value of 2(e)-Delta/DeltaCt in AML cases analyzed was 540, range 1.5-5043. We found that MBN is expressed at lower levels in AML than in healthy volunteers. The mean value of 2(e)-Delta/DeltaCt was 13 and 35 respectively. It increases in AML FABM2 (mean value of 2^-ΔΔCt :1051) and FABM3 (mean value of 2^-ΔΔCt:251) and in AML FABM4 (mean value of 2^-ΔΔCt:510) and it decreases again in FABM5 (mean value: 218) and FABM6 (mean value:49). Interestingly, patients affected by AML FABM2 and FABM4 with the presence of the t(8;21) and inv(16) showed significantly higher values of MBN respect to the same FAB subtypes with normal karyotypes. The mean value of 2^-ΔΔCt obtained in BM samples from patients with AML1/ETO-positive FABM2 AML compared to AML1/ETO-negative FABM2 cases was 1522 vs 164 (p=0.0001). Similar differences were detected by analyzing FAB M4 cases with and without the CBFB/MYH11 fusion transcript (mean value of 2^-ΔΔCt :961 vs 150) (p=0.0002). The MBN overexpression was detected in 60% of PB samples from RA patients (mean value: 10, range 3-268), and in all the cases of RAEB (mean value 201: range:128-803) and secondary AML (mean value 589, range 207-7131). Moreover, in these patients we could demonstrate a good correlation between the percentage of blast cells and MBN transcript amount. These data allow to demonstrate that MBN gene may probably play an important role in the leukemic transformation. The quantitative assessment of MBN expression can be a useful marker of leukemic and myelodysplastic
hematopoiesis. Finally, MBN could probably represent a good leukemia antigen for an immunotherapeutic approach.

PO-037
THALIDOMIDE ALONE OR IN COMBINATION WITH OTHER AGENTS FOR THE TREATMENT OF 248 PATIENTS WITH MYELODYSPLASTIC SYNDROMES (MDS)
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The last decade has been one of increasing interest and activity devoted to a better definition of the pathologic basis of MDS as well as identification of novel therapeutic targets in these diseases. Thalidomide was chosen for clinical trials in MDS for its three important properties: the anti-TNF, anti-angiogenic and immune-modulatory effects. A total of 248 MDS patients (pts) have received thalidomide (between 100 and 400 mg orally daily) either alone (83 pts) or in combination with other agents (165 pts) at the Rush MDS Center. All pts were symptomatic and needed to be treated. The mean age on thalidomide protocols was 67±11 years and 160 pts were males. As per FAB groups: RA 89, RARS 41, CMML 13, RAEB 86, RAEB- t 19. According to IPSS score: low-risk 40, Int-1 126, Int-2 53, high risk 29. Responses were assessed using the International Working Group criteria (IWG). Overall, of 248 MDS pts 52 (21%) obtained an hematologic response. A survival analysis shows that the 52 pts who responded have a better survival compared to pts. who did not respond to therapy (p=0.0002, log-rank test). When the pts received thalidomide as a single agent (83 MDS pts, 49 with RA/RARS, 56 low or Int-1 IPSS) we documented 21 (18%) responses (with 10 pts acquiring transfusion independence after a median of 16 weeks). There were some bi- and tri-lineage responses but the most hematological responses were restricted to the erythroid series. The majority of responders belonged to the refractory anemia (RA) or RA with ringed sideroblasts (RARS) categories. The same results were obtained when thalidomide has been combined with ciprofloxacin and dexamethasone (66 MDS pts, 49 with RA/RARS, 53 low or Int-1 IPSS, overall responses 17%). In other three studies thalidomide has been combined with other antineoplastic agents: thalidomide plus arsenic trioxide-ATO (28 MDS pts, 18 with RAEB/RAEB-t), thalidomide plus topotecan (45 MDS pts, 39 with RAEB/RAEB-t) and thalidomide plus etanercept (26 MDS pts, 3 with RAEB/RAEB-t). Overall in these protocols we documented 25 (25%) responses both in the RAEB/RAEB-t and in the RA/RARS cases; however the duration of response in RAEB and RAEB-t pts was shorter than in those with RA or RARS. Biological studies showed that arsenic trioxide and thalidomide combination produces multi-lineage hematological responses particularly in MDS pts with inv(3)(q21q26.2) and high pre-therapy EVI1 expression. These data, taking into account the differences of the population in the various studies, show that about 20-25% of MDS pts, with any FAB or IPSS score, can achieve a response to thalidomide alone or in combination. However only the pts with a low IPSS score or a low grade MDS (RARS or RA) are able to maintain this response and can reach a better survival; instead the pts with RAEB and RAEB-t have shorter response duration and no clear advantage on survival. Recently more potent and effective thalidomide analogs (such as CC5013-Revimid) have been introduced into clinical trials at the Rush MDS Center with very promising results (higher hematologic/cytogenetic responses and better tolerance compared to thalidomide) especially in pts with low and Int-1 risk MDS and in those with a del (5q) cytogenetic abnormality.

PO-038
INCIDENCE, CLINICAL-BIOLOGICAL FEATURES AND PROGNOSTIC SIGNIFICANCE OF DEL(5Q) IN PATIENTS WITH MYELODYSPLASTIC SYNDROMES
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An interstitial deletion of the long arm of chromosome 5, del(5q), is the most common deletion in patients with myelodysplastic syndromes (MDS), being observed in about 10-15% of patients. The cytogenetic defect is associated with macrocytic anemia, normal or elevated platelet number and peculiar morphological features, especially hypolobulated megakaryocytes. These last are so typical to be highly predictive of the del(5q) syndrome. From a prognostic point of view patients with the del(5q) syndrome have a significantly better clinical outcome than those with other karyotype abnormalities. Recently, the WHO committee for the classification of neoplastic diseases has recognized del(5q) syndrome as a separate entity. The aims of the present study were to establish the incidence of the del(5q)
in our series of 376 consecutive MDS patients, to correlate the defect with peculiar morphological features, to determine whether the breakpoints of the interstitial deletion, the presence of one additional defect and an increased medullary blast count affect overall survival (OS) and event-free survival (EFS). Cytogenetic analyses were carried out at diagnosis on bone marrow cells with a trypsin–Giemsa banding technique. Metaphase cells were obtained from short-term unstimulated cultures. Whenever possible at least twenty metaphases were analysed and ten fully karyotyped. An isolated del(5q) was discovered in a 32 patients, while a del(5q) plus one additional defect in 13. The median age of single del(5q) patients was 60 years (35–80 years). Male to female ratio was 1:1, but it changed to 1:1.7 when only refractory anaemia (RA) and refractory anaemia with ringed sideroblast (RARS) were examined. Twenty patients were classified as RA and RARS, 8 as refractory anaemia with excess of blasts (RAEB), 2 as RAEB in transformation and 2 as chronic myelomonocytic leukemia (CMML). The 20 patients with less advanced MDS showed erythroid hypoplasia and hypolobulated megakaryocytes as their most prominent morphologic features. In particular the former morphological finding was discovered in about 50% of patients. Considering del(5q) plus one additional defect four patients were classified as RA, 4 as RAEB, 2 as RAEB-t and 2 as CMMML. The median survival for patients with an isolated del(5q) was 138 months, while that of patients with an increased medullary blast count or with del(5q) plus one additional abnormality was 38 and 50 months respectively. When we compared patients with an isolated del(5q) versus those with del(5q) plus one additional defect death rates were 7.5 (95% confidence intervals, CI=2.9–19.8) vs 25.6 (95% CI=12.2–53.9) with an hazard ratio of 0.2 (95% CI=0.1–0.8) and death/progression rates were 11.5 (95% CI=5.1–25.8) vs 33.6 (95% CI=17.5–64.6) with a hazard ratio of 0.3 (95% CI=0.1–0.8). Patients with single del(5q) presented a significantly better OS and EFS in comparison to those with del(5q) plus one additional defect (p values= 0.01 and 0.02). No difference in OS and in EFS was seen in relation either to the different breakpoints of the interstitial deletion or to the amount of dysplasia. Death occurred in 8 patients with an isolated del(5q) and in 7 patients with del(5q) plus one additional defect, while leukemic evolution occurred in 11 and in 9 patients of each group respectively. In addition we could not segregate patients with single del(5q) from those belonging to the good-risk cytogenetic category as defined by the International Prognostic Scoring System (IPSS). In fact OS and EFS for both patient groups were similar. The same occurred when patients with del(5q) plus one additional defect were compared to those belonging to the IPSS intermediate-risk cytogenetics category. In conclusion our study strengthens the WHO definition of del(5q) as a separate entity within MDS subgroups but also suggests that patients with an otherwise typical 5q-syndrome but with an increased blast count or with additional defects should not be included within this entity.

**PO-039**

**PAROXYSMAL NOCTURNAL HEMOGLOBINURIA PATIENTS DISPLAY HIGH FREQUENCY OF CIRCULATING T LYMPHOCYTES EXPRESSING ACTIVATING ISOFORMS OF INHIBITORY SUPERFAMILY RECEPTORS**

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Recently, it has been reported that a minor subset of T lymphocytes express at the cell surface KIR and/or CLIR members of the Inhibitory Receptor Superfamily (IRS). These T cells would represent chronically stimulated memory cells expanded during viral infection or autoimmune responses. Paroxysmal nocturnal hemoglobinuria (PNH) is a clonal disorder of the hematopoietic stem cell (HSC) characterized by deficiency of glycosylphosphatidylinositol (GPI) membrane-linked proteins. It has been proposed that normal HSC are selectively eliminated by autoreactive T cells, while PNH HSC can escape this killing and thus survive and expand. The identity of autoreactive T cells and their target are still elusive. We have analyzed the surface expression and function of IRS members in T cells of PNH patients. We found that the proportion of KIR+ or CLIR+ cells within CD3+ cells was consistently higher in PNH patients than in healthy donors. In addition, the ratio between CD3+KIR+ and CD3−KIR+ or CD3−CLIR+ and CD3−CLIR+ was >1 in 8 out of 12 PNH patients, whereas this ratio was >1 only in 3 out of 30 healthy donors. This indicates that, beside by the increase of CD3+IRS+ cells, PNH patients are characterized by a decrease of CD3−IRS− Natural Killer cells. A minor fraction of CD3+IRS+ T cells express TCR γδ (belonging to the Vδ2 subset) and the remaining large fraction of these cells was TCR γδ+. PNH CD3+IRS+ T cells were characterized by a powerful cytolytic activity when triggered through the engagement of KIR or CLIR receptors. This indicates that CD3+IRS+ T cells express IRS belonging to the activating type. Clonal analysis revealed that in the large majority of T cell clones
derived from PNH patients the engagement of either KIR or CLIR elicited activation of cytolyis, while CD3+IRS+ T cells from healthy donors expressed IRS of the inhibiting type. The ligation of IRS on CD3+IRS+ T cell clones of PNH patients induced a strong production of TNF-α and IFN-γ. Finally, GPI- cells were less sensitive than their GPI+ counterpart to CD3+IRS-mediated killing. Altogether these findings suggest that CD3+ T cells expressing the activating isoforms of IRS are increased in PNH patients and thus they may include auto-reactive effector cells involved in the pathogenesis of this disease.

PO-040
LARGE GRANULAR LYMPHOCYTIC-LEUKEMIA, PAROXYSMAL NOCTURNAL HEMOGLOBINURIA AND APLASTIC ANEMIA: T-CELL CLONALITY BY TCR ANALYSIS SUPPORTS AN UNIQUE PATHOGENIC MECHANISM

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Large granular lymphocytic (LGL)-leukemia is characterized by expansion of phenotypically and morphologically distinct lymphocytes; paroxysmal nocturnal hemoglobinuria (PNH) is a non-malignant clonal disease of hematopoiesis defective in surface expression of glycosylphosphatidylinositol-anchored proteins; idiopathic aplastic anemia (AA) is a putatively immune-mediated attack of hematopoiesis. All these conditions are characterized by marrow failure, involving one or more hematopoietic lineages. We investigated the T-cell compartment by T-cell receptor (TCR) analysis in patients with these diseases, seeking dominant T-cell clones. Flow cytometry analysis of the TCR; chain was combined with molecular techniques for precise analysis of the complementarity determining region 3 (CDR3), which determines the antigen specificity of T-cells. Clonal populations were finally characterized by sequencing of the TCR-CDR3. We analyzed peripheral blood lymphocytes of a total of 108 patients (45 with LGL-leukemia, 24 with PNH and 39 with AA). Flow cytometry analysis of the TCR-V; usage demonstrated a massive expansion of CD8+ T-cells harboring a single TCR-V; subset in most LGL cases; few exceptions showed expansion of two V families. In contrast, usually PNH and AA patients showed an oligoclonal pattern of expansion, most patients having two or three over-utilized V; subsets. Some PNH cases showed extreme expansion of one TCR-V; subset, resembling what observed in LGL patients. The CDR3 pools from the expanded V; subsets were amplified by RT-PCR using a common constant (C) and the specific V primers; CDR3 size analysis (spectratyping) showed predominant peaks in all CD8 expansions, regardless the specific disease. For confirmation of clonality, V families showing a skewed CDR3-length pattern were cloned in bacteria and single colonies were sequenced. As expected, clonality was found in all LGL cases, with an identical CDR3 sequence generally obtained from more than 50% of colonies; in a few cases, subdominant clones were also demonstrated, suggesting that more than one clone may account for the LGL expansion. CDR3 pools of expanded CD8 V; subsets from PNH and AA patients showed high level of redundancy, and several CDR3 clonotypes were identified. Clonotypes were all patient-specific, with no preferential usage of particular V or J; furthermore, protein alignment algorithm did not show any structural homology, likely as result of the extreme HLA-class I heterogeneity. However, in two AA patients sharing 3 out of 4 class I antigens, clonotypes were almost identical (98% homology), strongly suggesting a public/semi-public HLA-restricted immune response. A longitudinal analysis was possible in some patients: four AA cases were serially studied after treatment with anti-thymocyte globulin-based immunosuppressive regimens. In all cases, great concordance between clonotype prevalence and blood counts was observed: dominant clones increased with progressive or not-responsive disease, while decreased after successful immunosuppression, eventually rising up in the presence of clinical relapse. Similar observation was possible in two LGL patients receiving cytoreductive agents; in contrast, two PNH patients who did not receive any treatment showed a progressive increase of the dominant clone. In conclusion, we documented that T cell clonality may be demonstrated in different marrow failure syndromes, regardless the primary disease. Theoretically clonality may result from the specific disease (such as a malignant LGL-clone); however we believe that it reflects a common pathogenic immune mechanism. According with this hypothesis, an antigen-driven immune response may underlie LGL, PNH and AA; the nature of the specific antigen(s), the efficiency of the target killing and additional biological features of the T cell clones may all influence the relative clinical manifestations of marrow failure versus T cell proliferation.
PO-041
FUNCTIONAL ANALYSIS OF T LYMPHOCYTES IN PAROXYSMAL NOCTURNAL HAEMOGLOBINURIA PATIENTS
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PNH is an acquired haematological disorder characterised by a clonal haemopoiesis derived from a defective stem cell. Clonal population exhibits deficiency of the GPI anchor on the cell membrane as a consequence of the alteration of an enzyme (located on the X chromosome) involved in the synthesis of the GPI anchor on the X chromosome. The GPI anchor is involved in the synthesis of asparagine on the X chromosome. The alteration of an enzyme (located on the X chromosome) involved in the synthesis of the GPI anchor on the cell membrane is a consequence of the alteration of an enzyme (located on the X chromosome) involved in the synthesis of the GPI anchor on the cell membrane as a consequence of the alteration of an enzyme (located on the X chromosome) involved in the synthesis of the GPI anchor on the cell membrane. The GPI anchor is involved in the synthesis of asparagine on the X chromosome. The alteration of an enzyme (located on the X chromosome) involved in the synthesis of the GPI anchor on the cell membrane is a consequence of the alteration of an enzyme (located on the X chromosome) involved in the synthesis of the GPI anchor on the cell membrane as a consequence of the alteration of an enzyme (located on the X chromosome) involved in the synthesis of the GPI anchor on the cell membrane. Data such as expression of cytokines and production of cytokine production are therefore of great interest. Interestingly, an altered response to activation stimuli of the normal polyclonal T lymphocyte compartment has been observed.

PO-042
FINAL RESULTS OF A PILOT STUDY EVALUATING THE ROLE OF DARBEPOETIN αα IN TREATING ANEMIA OF PATIENTS WITH LOW-INTERMEDIATE RISK MYELODYSPLASTIC SYNDROMES
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Darbepoetin-α (DPO) is a molecularly modified erythropoietin, characterized by the presence of augmented siaiylated carbohydrate content in its structure which permits a prolonged serum half-life and a possible increased in vivo biologic activity. DPO is currently licensed in Italy for the treatment of renal and chemotherapy-induced anemia. However, no published data are so far available about the role of DPO as single agent in anemic patients with myelodysplastic syndromes (MDS). Here we report the conclusive results of a pilot study in which we investigated the safety and the efficacy of DPO in MDS. Nineteen anemic patients (Hb < 9.5 g/dL, twelve transfusion-dependent) with low-to-intermediate-1 risk MDS according to the International Prognostic Scoring System (IPSS) were entered into this study. Thirteen were males and 6 females. Mean age was 63.7 years (range 42-84). According to WHO classification, there were four refractory cytopenias with trilineage dysplasia (RCMD), seven refractory anemias (RA), four RA with ring sideroblasts (RARS), one of which with multilineage dysplasia (RCMD-RS), three RA with excess of blasts type I (RAEB-1) and one 5q– syndrome. Two patients had a moderate degree of renal failure and two were MDS secondary to chemotheraphy for solid tumors. Five patients had previously received r-EPO without significant improvement of Hb levels or transfusional needings. All patients received DPO (Nespo, Dompe’-Biotec, or, alternatively, Aranesp, Amgen, Milan, Italy) at the dose of 150 mcg s. c. once-a-week (q. w), for at least 12 weeks, without additional therapies. Such a dose corresponded approximately to a mean of 30,000 U per week of recombinant αα or β r-EPO, considering the median weight of our patients (64.8 kg, range 54–85). The drug was kindly provided free of any charge by both producing Companies, on a compassionate basis therapeutic program. Local Ethic Committee approved the study and patients gave written informed consent. All patients completed 12 weeks of therapy at least. Fourteen patients, including all subjects who had previously received r-EPO, did not show any improvement in Hb levels or transfusional support and interrupted the study. Five patients (3 RA, 1 RARS and the patient with 5q- syndrome) responded to DPO (overall response 26.3%; C. l. 95%: 21%-38%; three major and two minor erythroid hematological improvements, according to IWG criteria). Three out of responders maintain stable hemoglobin levels > 9.5 g/dl after 11, 12, and 20 months of treatment, respectively. In two of them DPO is currently given every two weeks. One patient had a drop in his Hb levels after eight weeks of DPO therapy, due to the development of iron deficiency and required substitutive therapy to achieve a new response. One patient lost the response after 16 weeks. The fifth responder died after 5 months, due to causes unrelated to DPO treatment. No relevant adverse events or significant side effects were recorded throughout the study period. In particular, no case of pure red cell aplasia (PRCA), thrombosis, leukemic evolution or uncontrolled hypertension was observed. All responders had baseline serum levels of endogenous EPO < 200 miu/mL (four < 100 miu/mL), no or very low red-cell transfusion requirement, no excess of blast in bone marrow.
marrow, a diagnosis of MDS performed no more than 4 months before treatment with DPO, a single lineage (erythroid) involvement. Our results suggest that DPO is safely given and may be effective in a proportion of MDS. Percentage and possible predictive factors of response appear to be similar to those obtained with r-EPO in these patients.

**Poster**

**MOLECULAR HEMATOLOGY I**

**PO-043**

**GENE EXPRESSION PROFILE OF CHRONIC MYELOID LEUKEMIA STEM CELLS**


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To obtain comprehensive information about the genes involved in BCR/ABL-dependent leukemogenesis we studied the gene expression profile of lin+CD34−, lin−CD34+, and lin+CD34+ hematopoietic stem cells (HSC) from 8 chronic myeloid leukemia (CML) patients and 6 normal donors using Affymetrix HG-U95Av2 GeneChip array. Molecular caryotyping and quantitative analysis of BCR/ABL transcript demonstrated that within lin−CD34− the% of leukemic cells are 32%, whereas leukemic cells represent 60% of the total lin−CD34+ cells. Hierarchical clustering analysis paired normal lin−CD34− to CML lin−CD34− cells, CML lin−CD34+ to CML lin+CD34+ cells and normal lin−CD34+ to normal lin+CD34+ cells. Comparison analysis performed with Affymetrix MAS 5.0 software revealed 370 genes differentially expressed in all CML HSC subpopulations when compared to normal counterparts. The up-regulated genes in CML samples were found belonging to the cell cycle, mitosis, DNA replication and DNA repair Gene Ontology (GO) categories, whereas immune response, defense response, antigen presentation and antigen processing GO categories are up-regulated in normal samples. BCR/ABL modulates expression of genes which are involved in cell cycle regulation, DNA repair, apoptosis, like bcl2 family members and malignant progression, like angiogenic cytokines. Moreover, in CML samples we found down-modulation of genes involved in antigen processing and presentation, so the ability of CML HSC to function as Antigen Presenting Cells (APC) may be compromised. This study expands the knowledge on the genetic programs of CML and may represent a source of potential targets for CML therapeutics.
NUP98 gene is one of the so-called promiscuous genes as at least 15 partners of translocations are known. It is involved in both de novo and secondary hematological malignancies such as acute myeloid (AML) and lymphoid leukemias (ALL), Ph-positive chronic myeloid leukemia (CML), T-cell non-Hodgkin's lymphomas. In order to pick up NUP98 rearrangements, we set up a FISH approach with DNA clones selected for NUP98 and in some cases for the partner genes. We collected twelve cases with a NUP98 translocation and the following diagnosis: 2 T-ALL with t(4;11)(q12;p15); one refractory anemia with excess of blasts and a cryptic ins(5;11)(q35;p15p14); three AML-M2 and one Ph + CML with t(7;11)(p15;11p15); one AML with t(8;11)(p11.2;p15); one secondary AML-M2 with t(10;11)(q22;p15); two AML-M2 with t(11;12)(p15;q13); and one AML-M4 with inv(11)(p15q22). NUP98 was investigated by FISH with clone RP5-1173K1 spanning exons 10 to 20 on der(8) and der(11). In one case of t(11;12) the t(5;11) showed the same breakpoint at 7p15 within RP5-1173K1 and CTC-549A4; 3/4 patients with NUP98 rearrangements in all cases resulting in three hybridization signals on normal 11, in's lymphomas. In order to pick up NUP98 rearrangements, we set up a FISH approach with DNA clones to study specific NUP98 translocations. These tools will be helpful to unravel the incidence and the clinical impact of NUP98 rearrangements.

Funding: This work was partially supported by CNR-MIUR, and FIRB.
their median age was 42 years (range 8–71). All the patients except five, who died during the induction chemotherapy, achieved a complete remission (CR) after a median time of 42 days (range 32–68). The median follow-up time is now 29 months (range 0–92). Twenty-three patients are in CR after a median follow-up time of 29 months (range 3–92), while seven have relapsed after a median follow-up time of 20 months (range 9–42). A second CR was achieved in all these last patients except in one who presented high hTERT transcript levels along with FLT3 ITD. This patient died just before performing an allogeneic bone marrow transplantation (Allo–BMT). Two other patients in second CR underwent an allo-BMT. One of them with high hTERT transcript levels and FLT3 ITD experienced a third relapse but succeeded in reaching a new CR after additional intensive chemotherapy. The other patient with low levels of hTERT transcript level and without FLT3 ITD is in an un-maintained CR 24 months from the transplant. A high white blood cell (WBC) count either at clinical diagnosis or at relapse was the only clinical parameter which was significantly correlated with high hTERT transcript levels (p=0.037). Mean hTERT transcript level were 6.4 (range:0.7–9.9) for patients with high WBC and 4.9 (range: 0.3–14.7) for those with normal WBC at the onset of the disease. High hTERT values were observed in five out of the six FLT3 ITD positive patients. Considering all the patients hTERT levels presented ups and downs during the follow-up. We evaluated whether hTERT values determined either at clinical diagnosis or at one hundred–eighty days from it could predict relapse. We found that relapse rate was 4.7% (95% confidence intervals, CI=0.6–33.0) for patients who at the onset of the disease had low hTERT transcript levels versus 15.9% (95% CI=6.2–42) for patients who had high levels. These last patients showed a hazard risk of relapse of 4.3% (95% CI=0.48–39) (p=0.14). These same significant correlations were found for hTERT values determined at one hundred–eighty days from the onset of the disease. At this time relapse rate was 6.8% (95% CI: 0.9–48) for patients with low hTERT values versus 21.7% (95% CI: 7.0–67) for those with high levels. These last patients showed a hazard risk of relapse of 3.5% (95% CI: 0.37–34) (p=0.23). In conclusion i) high hTERT values are significantly correlated with high WBC count; ii) are more often seen in patients with FLT3 ITD; iii) are predictive of relapse independently of whether they are determined at diagnosis or at one hundred–eighty days from it.

**PO-046**

**EPIDEMIC EVENTS INVOLVED IN THE TRANSCRIPTIONAL SILENCING OF RA-TARGET GENES IN ACUTE MYELOID LEUKEMIA**

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Aberrant recruitment of HDAC activities favoring hypermethylation of target promoters underlies the pathogenetic action of AML-associated fusion protein AML1/ETO. Recent studies indicate that HDAC are present in complexes containing DNA-methyltransferase (DNMT’s) and methyl-CpG binding domain proteins (MBDs) to remodel chromatin and link deacetylation-mediated gene silencing to DNA methylation. Our previous observation indicated that a transcriptional repression of RA-signaling pathway underlies the pathogenesis of non APL AML–M2 and AML–M4. Using Southern blot analysis of genomic DNA and by methylation-specific PCR (MSP) we found that the RARβ2 promoter region containing the β-RARE and transcription start site is methylated in 7/9 AML–M2, 9/10 AML–M4 and in 6/8 AML1/ETO positive samples. The region located in the 5’portion of the exon 1 of RARβ2 is methylated in 9/9 AML–M2, 8/10 AML–M4 and 8/8 AML1/ETO positive samples. Neither of these RARβ2 regions is found methylated in CD34+ normal hemopoietic precursors. RARβ expression is detectable in normal CD34+ cells but not in any of the 24 AML cases analyzed. We analyzed whether the expression of AML1-ETO into hematopoietic progenitors induces repression of RA-signaling pathway by affecting the methylation status at RA-target genes. By using AML patients blasts and cell lines carrying an endogenous AML1/ETO (Kasumi and SKNO) or stably transfected with an HA tagged AML1/ETO (U937–AE) as cell model system our preliminary results indicates that: (i) AML1/ETO is present on AML1 (p14arf) and RA (RARβ2) target gene promoters complexed with DNMT and MBD activities as shown by chromatin immunoprecipitation (ChIP) assay; (ii) in the absence or in the presence of RA, the expression of AML1–ETO down–regulates in a dose–dependent manner the transactivation of a transiently transfected 5Kb RARβ promoter; (iii) the expression of AML1–ETO induces hypermethylation of the promoter/exon1 region of RARβ2 gene; (iv) both histone deacetylase and DNA methyltransferase inhibitors relieve the transcriptional repression of RA target genes and restore the differentiation response
of AML blasts to RA. In summary this preliminary results suggest that an aberrant hypermethylation at RA target gene is present in non APL AML blasts. In addition, the expression of AML1/ETO affects the methylation status of RA-target genes thus extending the rationale for a transcriptional therapeutic approach in AMLs.

**PO-047**

**MEK1 INHIBITION AND ARSENIC TRIOXIDE COMBINED TREATMENT INDUCES APOPTOSIS OF LEUKEMIC CELLS BY MODULATION OF P73 PROTEINS**

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Whereas the role of p53 in stress responses is well established, recent advances strongly support a pivotal role for the p53 paralog p73 not only in primary acute myelogenous leukemia (AML) blasts. Furthermore, we observed that combination of MEK/ERK pathway inhibitors with arsenic trioxide (ATO) enhances ATO induced apoptosis not only in primary acute promyelocytic leukemia (APL) blasts but also in primary blasts of other AML subtypes. To better understanding the mechanisms of this successful combination, we studied the behaviour of p73-p53AIP1 pathway in primary acute myelogenous leukemia (AML) blasts. Additional analysis of sub-G1 apoptotic NB4 and K562 cells after 72 hours of treatment with MEK1 inhibitor PD184352 (1 µM) and ATO was significantly diminished in cells transfected with control siRNA. These findings indicate that p73 is a major determinant of PD+ATO efficacy in leukemia cells carrying an inactive p53, and suggest that modulation of p73 proteins expression and/or function might represent in the future a new molecular target for leukemia treatment.

**PO-048**

**SNP MINING BY NANOGEN DEVICE: A SENSITIVE TOOL FOR THE IDENTIFICATION OF BCR/ABL MUTATIONS IN PH+ PATIENTS**

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**Background:** it was recently shown that resistance to Gleevec (STI571) in patients with CML or Ph+ALL, is associated with different single amino acid substitutions in distinct positions, known to be important for STI571 binding within the ABL kinase domain. The presence of mutations pre- and post-Gleevec treatment is currently included in the laboratory follow-up of CML and Ph+ALL patients receiving that drug. However most of the current available methods are either not enough sensitive or time-consuming. **Aim:** to test the feasibility to use the SNP mining by Nanogen device for the rapid identification of BCR/ABL mutations. **Methods:** Nanogen (San Diego, CA, USA) developed a method for the de novo discovery of genetic variations, including single nucleotide polymorphisms (SNPs) and mutations, on microelectronic chip devices. The method combines the features of electronically controlled DNA hybridization on open-format microarrays, with mutation detection by a fluorescence-labeled mismatch-binding protein. Electronic addressing of DNA strands to distinct test sites of the chip allows para-
allel analysis of several individuals. Vice versa, several SNPs/mutations can be tested in few individuals. This microelectronic chip-based mutation discovery assay may substitute time-consuming sequencing studies and will complement existing technologies in genomic research. Results: in a preliminary series of experiments, we demonstrated that a point mutation in ABL could be identified in a 10^4 BM sample dilution of a single patient resistant to Gleevec. The analysis of a larger patient series by parallel cloning/sequencing and SNP mining is still in progress. Conclusion: for the sensitivity and the short time required for the analyses of different patients samples, or multiple time points during follow-up, the Nanogen device represents an alternative to DHPLC or cloning, for the rapid identification of BCR/ABL mutations and monitoring of BCR/ABL mutations during Gleevec treatment.

**PO-049**

**FUNCTIONAL ANALYSIS OF THE PAX5/TEL CHIMERIC PROTEIN**

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**Background:** we previously cloned the PAX5/TEL chimeric gene, originated from the translocation t(9;12)(q11,p13) in an ALL adult patient. Recent data indicate that PAX5/TEL fusion defines the cytogenetic entity dic(9;12)(p13;p13). PAX5/TEL is likely to be an aberrant transcription factor, resulting from joining the 5' region of the partner gene PAX5 (a transcription factor essential for B cell development) to the 3' region of TEL/ETV6 (Ets-family DNA binding domain). Aim of the study was to investigate the functions of the PAX5/TEL chimeric protein as a potential oncprotein. **Methods:** we have cloned the FLAG-full length chimeric PAX5/TEL cDNA in the retroviral vector pMSCV-IRES-GFP (MigR1). NIH3T3 murine fibroblast and IL-3 dependent murine proB Ba/F3 cells lines were transduced with the retroviral construct to analyze subcellular localization and transforming activity of PAX5/TEL. **Results:** immunofluorescence analysis showed a specific nuclear localization of the chimeric protein in NIH3T3. Soft-agar colony forming assay of NIH3T3 infected with MigR1-PAX5/TEL did not show a significant transforming activity of the chimeric protein. IL-3 dependent murine proB Ba/F3 cells infected with MigR1-PAX5/TEL showed modulation of cellular growth rate; single subclones expressing the chimeric protein showed a decreased growth respect to control subclones. In addition, the expression of PAX5/TEL in Ba/F3 did not induce IL-3 growth independence. In summary, preliminary results did not show oncogenic activity of the PAX5/TEL fusion. Further analysis are needed to evaluate the functional role of the chimeric PAX5/TEL protein in Ba/F3 and other hematopoietic cell lines.

**PO-050**

**EPIGENETIC MODIFICATIONS IN A T(8;21) ACUTE MYELOID LEUKEMIA CELL LINE CAUSED BY ADMINISTRATION OF THE HDAC INHIBITOR D1**

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The acute myeloid leukemia (AML) cell line Kasumi-1 is characterized by the translocation t(8;21), manifested with the expression of AML1/ETO fusion protein, responsible for HDAC recruitment, determining transcriptional repression of target genes involved in myeloid maturation. We recently demonstrated that sodium butyrate and the stable prodrug xylitol butyrate derivative (D1) are able as single agents to restore histone acetylation and granulocytic maturation in Kasumi-1 cell line, as well as in primary AML blasts. D1 differentiative effects were confirmed by increased membrane expression of CD11b and CD15, decrease of CD34 and reduction of blasts absolute number. These effects were paralleled by massive induction of apoptosis as well as reduction of cell number. Acetylation and methylation of specific lysine residues on histone H4 and H3 has been associated with transcriptional regulation. The acetylation pattern of lysine residues of histone H4 was investigated. K5 and K16 residues were not acetylated in the absence of D1 and their acetylation strongly increased after 6 h D1 administration. K8 and K12 residues showed a basal acetylation which was further increased although not markedly. Lysine methylation of histone H3 was evaluated. D1 determined a reduction of di-methylated K9 H3, related to gene silencing. Di-methylated K4 H3, related to both gene silencing and transcriptional activation, was unmodified after D1 administration. These histone modifications paralleled D1-induced transcriptional activation of AML1/ETO target genes. The cyclin-dependent kinase inhibitors (CDKIs) belonging to the Cip/Kip family are known to be involved in cell cycle as well as apoptosis and cancer. D1 determined an increase of p21/cip1 expression and a decrease of p27/kip1. Appearance of a conspicuous amount of p57/kip2, an as yet uncharacterised oncosuppressor,
Chronic myeloid leukemia (CML) and acute lymphoid leukemia (ALL) are the most common Philadelphia chromosome positive leukemias in adults and children, respectively. More Philadelphia chromosome positive CML and ALL have treatment options than others like Hydroxyurea, Interferon and Gleevec. However, monitoring of therapy is very necessary to know the response of the patients to anti-leukemic therapy. Introduction of molecular biology techniques like PCR etc. revolutionized the research related to biomedical sciences. As single BCR-ABL fusion transcript acts as oncogene in Philadelphia chromosome positive leukemia in adults and children, respectively. More Philadelphia chromosome positive CML and ALL have treatment options than others like Hydroxyurea, Interferon and Gleevec.

After 72-96 h treatment with D1 was observed. The maturation effects of D1 were further investigated at molecular level. D1 was found to modulate the expression of transcription factors important for terminal cell differentiation. In particular, C/EBP epsilon, PU.1, c-jun, AML1/ETO as well as C/EBP alpha expression was modified after exposure to D1. These results suggest that HDAC inhibition determines profound gene remodelling through specific epigenetic modifications.

**PO-052**

**E-CADHERIN PROMOTER IS HETEROGENEously HYPERMETHYLATED IN BC-CML CELLS**

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Classical cadherin adhesion molecules are fundamental determinants of cell-cell recognition that function in cooperation with the actin cytoskeleton. They form a superfamily of molecules mediating calcium dependent cell-cell adhesion (N-cadherin, T-cadherin or CDH13, E-cadherin). E-cadherin serves as a widely acting suppressor of invasion and growth of epithelial cancers, and its functional elimination represents a key step in the acquisition of the invasive phenotype for many tumors. Moreover E-cadherin can negatively regulate, in an adhesion-dependent manner, the activation of divergent classes of receptor tyrosine kinases (RTKs). Hypermethylation is a frequent mechanism for silencing tumor suppressor genes. This is a potentially reversible epigenetic change, and it is the target of a novel class of anticancer compounds with demethylating activity. Chronic myeloid leukemia (CML) cells show a deficient beta1 integrin-mediated adhesion to stroma and are unresponsive to integrin mediated inhibition of proliferation. Defective adhesion properties could directly be due to bcr/abl protein effects on actin cytoskeleton and to its tyrosine kinase activity, but could also be determined by lack of expression of cadherins due to promoter hypermethylation, a phenomenon observed in CML for abl, calcitonin 1 gene and bcr sequences. Bi-allelic hypermethylation of the E-cadherin gene CpG island is common in acute leukemia, but its role in CML progression has not yet been elucidated. The methylation status of E-cadherin gene and its protein expression were evaluated. We investigated whether hypermethylation in the CDH11 promoter region was present in chronic phase and blast crisis (BC) CML primary cells and whether it could be modified by *in vivo* imatinib treatment. We also analyzed the effect of hypomethylating agents on the re-expression of E-cadherin. Cells were obtained after informed consent from patients with CML at diagnosis and after imatinib treatment and from patients with myeloid CML-CB. The analysis was performed by methylation specific PCR (MSP). MSP discriminates unmethylated alleles on the basis of DNA sequence alterations after bisulfite treatment of DNA, which converts only unmethylated cytosines to uracils. Sodium bisulphite genomic sequencing was used to fully characterize the methylation patterns of the CpG island associated with the E-cad-
herin gene promoter. Hypermethylation of CDH1 promoter was present in 60% of BC-CML, but only in 10% of CML chronic phase. Cells obtained after imatinib treatment did not show a modified pattern of methylation. Genomic bisulfite sequencing of CML cells confirmed dense methylation across the CpG island. Downstream signalling mechanisms, including the activation of STAT3 and Akt were examined by Western blot, together with E-cadherin protein surface expression indicating lack of activation of signalling molecules and defective expression of the protein in the methylated samples.

**PO-053**

**P210 BCR-ABL TYROSINE KINASE INHIBITS APOPTOTIC CELL DEATH THROUGH MULTIPLE PATHWAYS PREVENTING EARLY MITOCHONDRIAL ACTIVATION**

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The resistance to apoptotic death has a key role in the illegitimate enlargement of chronic myeloid leukemia (CML) hematopoiesis over its normal counterpart and in the genetic instability that drives clonal evolution of bcr-abl-rearranged myeloid progenitors towards the fully transformed phenotype of blast crisis. It results from multiple events preventing pro-apoptotic pathways (Trail–DR4, Fas–ligand and Bax induction, Bax and Bad translocation to the membranes of subcellular organelles such as mitochondria or endoplasmic reticulum, cytochrome-c release and caspase-3 activation) and/or enhancing pro-survival signals such as Bcl–2, Bcl–xl and survivin, and is mostly conditional upon the tyrosine kinase of p210 bcr-abl fusion protein. Our study addressed the matter of p210 bcr-abl tyrosine kinase effects on expression levels and subcellular locations of proteins that trigger apoptotic death in response to extrinsic or intrinsic signals by antagonizing anti-apoptotic Bcl-2 and Bcl–xl at the mitochondrial membranes. To the purpose, in individual cell clones generated from the murine myeloid 32D cell line transduced with a temperature-sensitive (ts) p210 bcr-abl construct (lacking the abl constitutive tyrosine kinase activity at the non-permissive temperature of 39°C) we investigated transcriptional induction and post-transcriptional modifications of pro-apoptotic signals in response to growth factor withdrawal, starvation, TNF–± and tyrosine kinase inhibitor STI571.Trail–DR4, Fas, Bax and Bim transcriptional induction, initiator caspase 8, 9 and 12 activation, Bad dephosphorylation, Bid cleavage and Bax and Bak aggregation at the mitochondrial membranes preceding citochrome-c release and executioner caspase activation are prevented by p210 bcr-abl tyrosine kinase through interactions with multiple transcription factors including Stat5, PI3K/Akt, Foxo3A and c-Myc. Our results may be helpful to design novel therapeutic strategies intended for targeting gene products relevant for CML progression in addition to p210 bcr-abl tyrosine kinase.

**Funding:** The study was supported by Università di Bologna (ex 60% and Progetti Pluriennali), Carisbo Foundation, Forlì–CesenaAll and CIB. GB is the recipient of a grant entitled to Mrs. Lalla Seràgnoli.
MAPKS ARE INVOLVED IN THE SURVIVAL AND DIFFERENTIATION OF A T(8;21) ACUTE MYELOID LEUKEMIA CELL LINE AND ARE MODULATED BY THE HDAC INHIBITOR D1

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Acute myeloid leukemia (AML) is a disease characterized by a block of maturation. Genes coding for core binding factors are rearranged in a considerable subset of AML cases and result in the altered interaction of CBF subunits with transcriptional co-regulators (NCoR/SMRT). Recruitment of HDAC is also altered in this subtype of AML, and a subsequent transcriptional repression of target genes involved in myeloid maturation is determined. We recently demonstrated that sodium butyrate and the stable prodrug xylitol butyrate derivative (D1) as single drugs restore histone acetylation and granulocytic maturation in the t(8;21)-positive Kasumi-1 cell line, as well as primary CBF-AML blasts. These effects are paralleled by massive apoptosis as well as reduction of cell number. The mitogen-activated protein kinases (MAPK) have been shown to regulate a wide variety of cellular processes, such as cell proliferation, differentiation and apoptosis. D1 induced ERK activation (peaks at 2-6h and 48-72h) and a transient activation of p38,JNK activity was also modulated following D1 treatment, with differences among the JNK isoforms. Specific inhibitors of the ERK, p38 and JNK pathways (PD98059, SB203580 and SP600125, respectively) were then used to investigate the role of MAPK in the biological effects determined by D1 administration to Kasumi-1 cells. Inhibition of ERK1/2 and JNK determined a significant decreased in cell number. P38 inhibition did not affect cell number while inhibiting the D1-induced decrease. Inhibition of ERK and JNK were ineffective in the D1-induced decrease. Decrease of cell number was caused by induction of apoptosis, as determined by the Annexin V test. In particular, ERK and p38 inhibition did not determine significant apoptosis. JNK inhibition determined a marked apoptosis which was similar to that induced by D1, while the treatment with both D1 and SP600125 induced an additive effect. Inhibition of the ERK and p38 pathways did not interfere with D1-induced apoptosis. These results were confirmed by western blotting evaluation of caspase-3 activation. Indeed, caspase-3 precursor decreased and the active caspase form appeared after a 24 h treatment with D1 and SP600125 alone, while the combination of D1 with SP600125 determined maximal effects. Caspase-3 activation was unchanged by the treatment with PD98059 or SB203580, alone or in combination with D1. PD98059 and SP600125 were able to induce a conspicuous maturation, as revealed by morphological analysis. These data were not supported by immunophenotype data. None of MAPKs inhibitors interfered with D1-induced maturation. It is worth pointing out that none of the above MAPK inhibitors modulated histone acetylation which occurs after D1 treatment of these cells. D1-induced modulation of MAPK, which seems to be involved in Kasumi 1 survival and maturation, suggests that the butyrate derivative exerts its action also via HDAC-unrelated pathways.

COVALENT MODIFICATIONS OF HISTONE H4 NH2-TERMINAL TAILS ASSOCIATED WITH P210 BCR-ABL TYROSINE KINASE

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Covalent modifications of the core histone tails, including acetylation, methylation, phosphorylation, ADP-ribosylation and ubiquitination govern the chromatin accessibility and create binding surfaces for protein recognition (such as the bromodomain for acetylated lysines and the chromodomain for methylated lysines), therefore dictating the rate of gene transcription. We investigated the impact of p210 bcr-abl tyrosine kinase of chronic myeloid leukemia (CML) on the acetylation and methylation at individual lysine residues within the NH2-terminal tails of histone H4, an integral component of transcriptional competence at the onset of S phase. To the purpose, we used RP-HPLC coupled with electrospray mass spectrometry (LCMS) to resolve the single H4 species (non-acetylated and mono-, di- or three-acetylated) and enzymatic digestion coupled with MALDI mass spectrometry to define the individual lysine residues involved in acetylation and methylation processes. In single cell clones generated from the murine myeloid 32D cell line transducing a temperature-sensitive (ts) p210 bcr-abl construct (that owns constitutive tyrosine kinase activity at the perch-
missive temperature of 33°C, but lacks it at the non-permissive temperature of 39°C) the inhibition of p210 bcr-abl tyrosine kinase induced by 1 µM STI571 was associated with a significant (p<0.001) reduction from 21% to 13% of three-acylated species of histone H4. Accordingly, a significant (p<0.001) increase of non-acetylated histone H4 species from 3% to 9% became apparent since 4th hour of exposure to STI571. The hyperacetylation status of histone H4 associated with p210 bcr-abl tyrosine kinase was ascribed to enhanced acetylation at the Lys5 and Lys12, whereas Lys20 and Arg19 appeared involved in methylation defects of ts p210 bcr-abl-transducing 32D cells, suggesting that these residues may represent, indeed, the preferential substrates for enzymes (histone deacetylases and methyl-transferases) that concurrently control histone covalent modifications and gene transcription. The significant reduction of transcription rates of several unrelated genes, including bcr-abl, following the exposure to STI571 of ts p210 bcr-abl-transducing 32D cell clones further supports a role for chromatin architecture in the pathogenesis and progression of Ph+ leukemias.

Funding: The work was supported by Università di Bologna (ex60% and Progetti Pluriennali), Carisbo Foundation, Forli-CesenaAIL and CIB. GB is the recipient of a grant entitled to Mrs. Lalla Seràgnoli.

PO-056
FIRST REPORT OF MULTIPLE POINT MUTATIONS IN ABL GENE ATP-BINDING DOMAIN CONFERRING PRIMARY IMATINIB RESISTANCE IN CHRONIC MYELOID LEUKEMIA PATIENT
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Imatinib (Gleevec, STI 571) is recently regarded as the most efficient therapy for CML patients. This drug directly inhibits the leukemogenesis by blocking ATP-binding situted of bcr-abl protein encoded by BCR-ABL oncogene; fusion gene resulted due to balanced translocation t(9:22)(q34; q11). Recently, point mutations have been detected in ATP-binding domain of ABL gene which disturbs the binding of Imatinib to this target, leading to complete or partial Imatinib resistance, depending upon the natures and location of mutations. We established to very sensitive allele specific oligonucleotide (ASO) PCR to detect mutations in to CML patient with no hematologic, cytogenetic or molecular response to an oral dose of 400 mg per day of Imatinib for nine months. We detected C944T and T1052C mutations. Mutation C944T causes complete resistance manageable by imatinib combination therapies while mutation T1052C causes partial imatinib resistance manageable by imatinib dose escalation. This is the first report of two different mutations conferring Gleevec resistance in the same patient at the same time. Previous studies of Gleevec resistance due to ABL ATP-domain mutations are based upon single mutation. Therefore, biological impact and clinical significance of two or mutations conferring resistance by different mechanisms in the same patient at the same time is still to be determined. However, it is postulated that imatinib resistance due to multiple mutations can lead to very complicated patterns of resistance which may be very difficult to manage clinically. We also conclude that Imatinib resistance in CML patients can be determined at earlier stages of Gleevec therapy by this technique.

PO-057
X-CHROMOSOME INACTIVATION PATTERNS IN WOMEN WITH PH-NEGATIVE CHRONIC MYELOPROLIFERATIVE DISORDERS
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It is generally assumed that chronic myeloproliferative disorders (CMDs) [i.e., polycythemia vera (PV), essential thrombocythemia (ET) and myelofibrosis with myeloid metaplasia (MMM)] result from clonal expansion of mutated hematopoietic stem cells. However, at least a proportion of patients with ET has been demonstrated to clearly exhibit polyclonal hematopoiesis with polyclonal patterns in granulocytes and platelets. In this study we have evaluated X-chromosome inactivation patterns (XCIP) in 191 Ph-negative CMDs female patients [45 with PV, 131 with ET and 15 with MMM] by the analysis of the human androgen receptor (HUMARA), phosphoglucerase kinase (PGK) and iduronate-2-sulphatase (IDS) loci. Based on granulocyte and T-cell cleavage ratios (CR) between alleles, the clonality status of hematopoiesis was defined as follows: a) polyclonal XCIP if CR < 3.0 for both granulocytes and T-cells; b) clonal XCIP if CR 3.0 for granulocytes and CR < 3.0 for T-cells; c) ambiguous XCIP if CR 3.0 for both granulocytes and T-cells. The initial screening revealed that 118 out of 191 (62%) patients were heterozygous for HUMARA, 17 out of 73 (23%) for PGK, and 11 out of 57 (19%) for IDS. Combining the approaches, 146 out of 191 (76%) CMD patients were informative. Within informative female patients, 29 out of 36 with PV (81%), 84 out of 98 with ET (86%) and 11 out of 12 with MMM (92%) displayed clonal or ambiguous XCIP. We used transcription-based clonality assays in all patients showing polyclonal XCIP in order to
analysed the transcribed HUMARA and/or IDS alleles on mRNA obtained from purified populations of platelets and/or granulocytes. Balanced Xcip were confirmed in 4 out of 5 analysable PV and in 9 out 11 analysable TE patients. The demonstration of polyclonal hematopoiesis in women with a typical clinical picture of ET has already challenged the dogma that CMDs derive from clonal proliferations of hematopoietic stem cells. Our findings demonstrate polyclonal hematopoiesis also in PV patients. Four women had unequivocal clinical features and met the diagnostic criteria for PV, but consistently showed polyclonal Xcip. This indicates that PV is heterogeneous with respect to lineage involvement. In some individuals, the abnormal clone might be restricted to erythroid cells and/or might be capable of sustaining an increased red cell production despite its limited size. The fact that all women with myelofibrosis with myeloid metaplasia showed clonal or ambiguous Xcip clearly indicates that this condition represents a true stem cell disorder in most instances.

**References**

formed clonal analysis of CP-CML female patients, using X-linked polymorphic marker HUMARA to confirm that also imatinib-induced complete cytogenetic responses are associated with the restoration of a true polyclonal haematopoiesis. Furthermore we analyzed the residual amount of p190 and p210 transcripts, in order to study the relationship between residual neoplastic clone and normal (i.e. polyclonal) haematopoiesis. Our results are referred to marrow sample performed after one year of imatinib mesylate therapy. We studied 14 CP CML females, enrolled in the Novartis-sponsored multi-institutional Phase II trials, who reached a complete cytogenetic response with Imatinib mesylate therapy. Nine patients had been previously treated with cidofovir, but this therapy was interrupted for intolerance (n.7) or lack of any cytogenetic response after one year of therapy (n.2). Five patients had received a short course of hydroxyurea. Before the start of Imatinib therapy all cases had a predominance of Ph positive metaphases in unstimulated bone marrow. Glivec was administered at the dosage of 400 mg/die. In 13 patients a sustained complete cytogenetic remission was documented in a median of 6 follow up samples (range 3-9). In one patient metaphases were available for study only in one sample, which however did not disclose Ph metaphases. FISH analysis was performed in every marrow sample during the follow up. Bcr/abl rearrangement. P210 transcript was detected by RT-PCR in each patient before starting therapy. Its persistence was documented in all patients during complete cytogenetic remission (in a median of 6 samples). After one year of therapy P190 transcript was found in 9 out of 14 patients during CCR while it was detectable only in two patients before the start of Imatinib therapy. The analysis of X-linked polymorphic marker HUMARA was performed after 12 months of imatinib therapy, during CCR. All patients were informative (i.e. heterozygous) for clonal analysis. All but one samples revealed a polyclonal pattern of X methylation after FISH analysis confirmed by densitometry analysis. The observation that 13 out of 14 patients exhibited a polyclonal pattern of X inactivation during complete cytogenetic response confirms the recent evidence that imatinib is able to restore a polyclonal haematopoiesis in CML patients. The present molecular study of patients in CP-CML in CCR induced by imatinib-mesylate discloses some clonal heterogeneity. Besides physiological haematopoiesis, which is largely predominant, as indicated by the analysis of X-linked polymorphic marker HUMARA, at least two subclones seem to coexist; the first expressing the p210 protein, the second expressing the p190. Furthermore, the monoclonal pattern observed in one patient of our series and the recent observation of the possible occurrence of clonal cytogenetic abnormalities in patients with CML in polyclonal remission after imatinib suggest that a neoplastic stem cell lacking bcr-abl rearrangement, may acquire further cytogenetic alterations different from Philadelphia chromosome. There is the possibility that imatinib therapy may prevent the expansion of Ph positive clones and that, in addition to stem cell genetic instability, other cytotoxic treatments may favour clonal evolution.

**PO-060**

**GENOMIC UNBALANCES ASSOCIATED WITH THE RESISTANCE TO IONIZING RADIATIONS OF A HUMAN OSTEOSARCOMA CELL LINE (SAOS) DETECTED BY COMPARATIVE GENOMIC HYBRIDIZATION (CGH-BASED TECHNIQUE)**

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Resistance to ionizing radiations (IR) is one major problem in the clinical management of osteosarcoma (OS) and marks the clonal evolution of tumor cells towards a more aggressive phenotype. Genome–wide screening techniques will be therefore helpful to uncover genomic unbalances eventually associated with specific characteristics of OS cells, including their radio- and/or drug–resistance. Here, we report on the results of a comparative genomic hybridization (CGH)–based technique to achieve informations on the genomic profile associated with radio-resistance in a human OS cell line, named SAOS, that lacks the p53-dependent radio-response. Radio-resistant SAOS subline was generated from the parental cell line following three rounds of low dose (10 Gy)/low dose rate (0.05 Gy/min) gamma irradiation performed at 24 hr intervals. Genomic unbalances associated with the development of radio-resistance were revealed by the co-hybridization of SpectrumGreen-labeled DNA from radioresistant SAOS and SpectrumRed-labeled DNA from parental cell line on AmpliOnc (GenoSensorTM Array 300) microarrays, representing the oncogenes and the tumor suppressor genes most frequently altered in human cancers (for the complete list of spotted targets see: Mao et al., Genes Chromosomes Cancer 35, 144-155,2002). The statistical analysis of obtained results was performed by normalized test–to–reference ratios of the included spots for each target calculated by the mass method according to a previously published work (Hattinger et al., Europ J Cell Biol 82,483-493,2003). The radio-resistance of SAOS OS cells was associat-
ed with the gain of the inherent DNA sequence copy number relative to 11 genes and no losses. In more detail, the amplification relative to thymidine kinase 1 (mapping at 17q25.2-q25.3), telomeric BAC and YAC regions (mapping at chromosomes 9 and 2, respectively) and EGR2 (a component of the mitochondrial apoptotic loop mapping at 10q21.3) was very significant, whereas the amplification relative to two telomeric sequences (mapping at 19p and 2p loci, respectively), to the chromosome segregation 1-like CAS (mapping at 20q13 locus), the cyclooxigenase 2 (mapping at 1q31.1), the ERBB2 sequence (located at the 17q21.1 locus) and the Myc (mapping at 8q24.12-q24.13 locus) showed a borderline statistical significance. Genomic unbalances associated with radio-resistance exhibited discrete differences compared with methotrexate-resistance, suggesting that OS cells utilize different pathways to repair the damage induced by reactive oxygen species or metabolic defects. Additional studies currently in progress on gene expression of parental and radio-resistant SAOS cells, performed on U133A microarrays from Affymetrix, will help to elucidate whether in OS lacking p53-dependent response to IR the radioresistance has specific genomic markers.

Funding: The study was supported by Università di Bologna (Progetti Pluriennali), Carisbo Foundation and Forlì-Cesena Al. GB is the recipinet of a grant entitled to Mrs. Lalla Seràgnoli.

PO-061
QUALITATIVE AND QUANTITATIVE MOLECULAR ASPECT IN ACUTE PROMYELOCYTIC LEUKEMIAS

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The acute promielocytic leukemia (APL) is a heterogeneous group of acute myeloid leukemia’s her expression is 10–15%, it is M3 for the FAB (French-American-British –Cooperative –Group classification. The APL is clinically Characterized from elevate sensibility at the acid trans-retinoic (ATRA) therapy. In this study we evaluated the quantitative and qualitative molecular investigation and the clinical utility in the exordium and follow-up phase, because of the elevated sensibility of Real-Time PCR, is possible recognize molecular relapse not visible with other morphologic markers. The increase of transcript copies is evidenced in this study. In exordus and follow-up phase, because of the elevated sensibility of Real-Time PCR, it is possible recognize molecular relapse not visible with other morphologic markers. The increase of transcript copies is evidenced by positive signals. In this way, is possible to use an appropriate therapeutic selection.

Funding: Studio realizzato con i finanziamenti dell’Assessorato Igiene e Sanità’ della Regione Sardegna

PO-062
IL-12 GENE EXPRESSION INTO AML-DERIVED DENDRITIC CELLS OVERCOMES T-CELL FUNCTIONAL IMPAIRMENT INDUCED BY LEUKEMIC MICROENVIRONMENT

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Acute myeloid leukemia (AML) cells may be differentiated into leukemic dendritic cells (AML-DC). AML-DC have superior immunogenicity than not-differentiated counterpart, but their antigen-presentation capacity within an inhibitory tumor microenvironment is unknown. As potent anti-tumor cytokine, interleukin (IL)-12 contrasts immune evasion by tumors. To test the effect of leukemic microenvironment on AML- DC/T interaction, T cells were incubated with AML-DC in presence of supernatant of human leukemic cell line, K562. As a result, T cell proliferation decreased and Th1 cytokine profile was lost. T cells were the target of this inhibition as demonstrated by reduced T-cell-derived IFN-γ production upon non-specific stimulation and by the lack of effect of leukemic supernatant on phenotype and cytokine production of CD40L-matured AML-DC. We then, transfected AML-DC with human IL-12 genes by using a non-viral method, nucleofection. Transfected AML-DC produced significant amount of IL-12, still showed DC-like phenotype and better allostim-
ulatory capacity than primary AML blasts and expressed leukemia-specific cytogenetic abnormalities. IFN-γ production by T cells cultured with IL-12-transduced AML-DC increased as compared to that obtained with mock-transduced DC. More importantly, T cells stimulated by IL-12-producing AML-DC in presence of inhibitory leukemic supernatant still maintained a Th1 cytokine pattern. In conclusion, IL-12 genes may be expressed into AML-DC by using a novel non-viral method which does not modify their phenotypical, cytogenetic and functional features. The constitutive production of IL-12 by AML-DC results in increased antigen-presentation capacity and counteracts the inhibition of leukemic microenvironment on T cells.

**PO-063**

**EVIDENCE OF THE OCCURRENCE OF NAD(P)H OXIDASE ACTIVITY IN HEMATOPOIETIC STEM CELLS: POSSIBLE INVOLVEMENT IN O2 SENSING AND ROS-MEDIATED DIFFERENTIATION SIGNalling**

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Despite of the growing interest in the field of stem cell research, promising important advance in the basic understanding of cell differentiation as well as in the cell-based therapeutic clinical application,1 a biochemical metabolic characterisation of this unique cell type is lacking. This study was aimed to partly fill such gap focusing attention on the terminal oxidative metabolism. The cell type chosen was human haematopoietic stem cell (HSC) mobilised from bone marrow by cytokine (G-CSF) treatment and collected from peripheral blood.2 The protocol of cell isolation based on positive immuno-selection by a monoclonal antibody raised to CD34 antigen (a specific surface marker of HSCs) resulted in never less than 99% of phenotypically homogeneous cell population. The main results obtained by an extensive analysis of the cell population revealed an inverse correlation between the mitochondrial content and the intensity of the signal attributable to the CD34 stem cell marker. In addition, analysis of the mitochondrial intracellular network of other cell types allowed to relate and compare the respiratory activity to the mitochondrial area. c) The small amount of mitochondrial respiratory complexes in HSCs was verified by differential spectrophotometric analysis on whole cell lysate and by blue-native 2D SDS–PAGE analysis of the oxidative phosphorylation complexes in isolated mitoplasts. d) A reexamination of the CN-insensitive endogenous respiration, amounting to about 50% of the overall endogenous respiration, revealed that this was completely abolished by DPI (a specific inhibitor of flavoenzymes) and sensitive to externally added catalase and/or superoxide dismutase, suggesting the involvement of a NAD(P)H oxidase-like activity converting O2 to O2 -. This was verified by the occurrence of ter-butyl isothiocyanide shiftable absorbance peaks at 425 and 558 nm, indicative of the presence of cytochrome b558, the NAD(P)H oxidase prosthetic group. d) reverse-PCR amplification of total RNA cell extracts followed by sequencing showed the expression in HSCs of membrane bound and cytosolic subunits of the NAD(P)H oxidase (gp91phox-NOX2, p22phox, p67phox, p47phox). Furthermore cross-immunoprecipitation analysis revealed the occurrence of an assembled complex and the phosphorylation state of the p47 cytotoxic subunit. Taken all together these results show that the mitochondrial oxidative phosphorylation capacity of the CD34+ hematopoietic stem cell is very low when compared with that of other cell types. This could be a consequence of the low energy demand of the G0-phase in which the resting HSC rely. The very low tension of O2 of the bone marrow stromal microenvironment (stem cell niche), could also be a factor conditioning the expression of the oxidative phosphorylation system. It is noteworthy, however, the presence of mitochondria able to locally generate and maintain a transmembrane potential as shown by the confocal microscopy analysis. This was particularly evident in a sub-population of the CD34 + cells apparently expressing lower level of the surface marker (whose progressive lost is indicative of commitment), suggesting a role of the mitochondrial oxidative metabolism in the early stage of HSCs differentiation. The novelty emerged from this study is the discovery of the presence of a NAD(P)H oxidase activity in HSCs (never reported before). Although both the catalytic and regulatory subunits of the NAD(P)H oxidase system are expressed and assembled, the activity, measured as DPI-sensitive oxygen consumption rate, is much lower than that of macrophagic cells where the NAD(P)H oxidase serves as a powerful oxygen producing bactericide system. Low active isoforms of the macrophagic NAD(P)H oxidase have,
however, been reported in other non macrophagic cells and it has been suggested their involvement in oxygen radical-mediated intracellular signalling. 3 It is tempting to suggest that following activation by external stimuli, the HSC NAD(P)H oxidase might be involved in a ROS-mediated intracellular signalling leading (or contributing) to cell differentiation. The nature of the external stimuli, the type of oxygen reactive species, the targets of the activated intracellular system are under investigation to validate our hypothesis.

References


PO-064

CARBOXY-TERMINAL FRAGMENT OF OSTEOGENIC GROWTH PEPTIDE REGULATES MYELOID DIFFERENTIATION THROUGH RHOA

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The carboxy-terminal fragment of osteogenic growth Peptide (sOGP10-14) is a pentapeptide with bone anabolic effects and hematopoietic activity. The latter activity appears to be largely enhanced by specific growth factors. To study the direct activity of sOGP10-14 on myeloid cells, we tested the pentapeptide proliferating/differentiating effects in HL60 cell line. In this cell line, sOGP10-14 significantly inhibited cell proliferation, and enhanced myeloperoxidases and NBT reducing ability. Moreover, it induced cytoskeleton remodelling and small GTP-binding protein RhoA activation. RhoA, which is known to be involved in the HL60 differentiation, mediated these effects as shown by using its specific inhibitor, C3. Treatment with GM-CSF had a comparable sOGP10-14 activity, and treatment with both growth factors showed enhanced effects. These results strongly suggest that sOGP10-14 acts directly on HL60 cells by activating RhoA signalling although other different possibilities cannot be ruled out.

PO-065

HB FEDERICO II, A NOVEL HAEMOGLOBIN VARIANT (β106 LEU→VAL) ASSOCIATED WITH A β-THALASSEMIA PHENOTYPE

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Hemoglobinopathies are monogenic disorders widespread overall, characterized by great heterogeneity in molecular pathology and encompassing the complex and partially overlapping groups of haemoglobin variants and thalassemia syndromes. So far, about 700 haemoglobin variants have been identified; some of them show decreased stability, due to the type and/or the location of the amino acid involved in the substitution. Several mechanisms for the decreased stability of a haemoglobin variant, giving rise to an extreme variability both of the instability level and the possible clinical manifestations, have been proposed. Although it is generally agreed that the clinical effects depend on the presence of an abnormal protein, it cannot be excluded that a DNA mutation could lead to transcription of an aberrant mRNA with decreased stability because of changes in the secondary structure. The aim of this study was to set up a method based on the noticeable and careful real-time PCR method to quantify mRNA levels of hemoglobin variants showing thalassemic features. β-thalassaemic mutations analysis was carried out on DNA and RNA obtained from leucocytes and reticulocytes respectively, collected from whole blood. RNA molecules measurement was performed on an iCycler instrument from Bio-Rad Laboratories using a common reverse and two allele-specific forward primers, in combination with SYBR Green I as the detection format. Recently, a patient showing a typical β-thalassaemic trait was referred to our laboratory for molecular characterization. Screening of the most common β-thalassemic mutations (β+87, β°6, β°11, β°6, β°11, β°6) in the Mediterranean population resulted negative. Sequence analysis showed solely the presence of a mutation at heterozygous level in the third exon of the β-globin gene, causing the substitution of Leu with Val residue (CTG→GTG) at codon 106 and producing a novel haemoglobin variant. This variant, which we named Federico II, was undetectable at the cation-exchange HPLC analysis, while only a small abnormal peak (7% of total haemoglobin) was revealed at the reverse-phase HPLC. To verify whether the extremely low level of this variant
was due to a reduced mRNA synthesis, measurement of the \( \beta \)-globin mRNA by real-time PCR were performed. We found that the \( \beta \)-globin mRNA levels of the proband were comparable to those detected in carriers of common \( \beta \)-thalassaemic mutations and about three-fold lower than those of a normal subject, thus confirming a quantitative \( \beta \)-globin gene expression defect in the proband. Furthermore, to assess that defective \( \beta \)-globin expression was associated to the mutant allele, evaluation of allele-specific mRNA expression performed in the proband revealed a strong disequilibrium in the expression levels of normal and aberrant mRNA species. The level of abnormal \( \beta \)-globin mRNA was about 20-fold lower than the normal one, thus indicating either a decreased transcription rate or instability of mutant mRNA molecules. Our date confirm and reinforce previous observations which indicate that substitutions at codon 106 could result in thalassemic or hemolytic phenotypes. Putative molecular mechanisms at the basis of the lowered expression rate, as well as protein stability and functionality features of this novel variant are under investigation.

**Poster**

MULTIPLE MYELOMA I

**PO-066**

**HIGH ACTIVITY OF CK2 SERINE-THREONINE KINASE SUSTAINS SURVIVAL AND PROLIFERATION OF MULTIPLE MYELOMA CELLS AND ITS BLOCKADE CAUSES GROWTH ARREST, APOPTOSIS AND ALTERED NFKB SIGNALING**


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Multiple myeloma (MM) pathophysiology is characterized by the aberrant activation of several signalling pathways triggered by external stimuli. CK2 (casein kinase II) is a ubiquitous serine-threonine kinase whose activity is elevated in proliferating and transformed cells. The role of CK2 in oncogenic transformation, apoptosis and cell cycle progression has recently become matter of intense research. Due to its functional connection with signalling molecules pivotal for plasma cell survival, such as those implicated in the TNF\( \alpha /NFKB\), IGF1/PI3K/AKT and Wnt/\( \beta \)-catenin pathways, CK2 is likely to play a central role in MM cell biology. We investigated the role of CK2 in MM plasma cell survival and cell cycle progression, in the regulation of the IkB/NFkB dependent pathway and in the modulation of MM cell sensitivity to chemotherapeutics. CK2 protein levels and enzymatic activity were analyzed in MM cell lines and highly purified CD138+ plasma cells from MM patients. To hamper CK2 function we used novel compounds that selectively inhibit CK2 activity and afterwards analyzed MM cell survival, apoptosis, cell cycle progression and MM cells sensitivity to chemotherapeutics. After CK2 block, we checked IkB protein levels at basal and TNF\( \alpha \) stimulated conditions and NFKB DNA binding by EMSA and transcriptional activity by luciferase assay. We found that CK2 protein is over-expressed and its kinase activity is increased in MM cell lines and purified CD138+ plasma cells from patients, as compared to control (resting peripheral blood lymphocytes and splenic B lymphocytes). Disruption of CK2 enzymatic activity with selective agents caused a dose-dependent cytotoxic effect as judged from increased apoptosis and cell cycle arrest in G2-M. Moreover, both the extrinsic
and the intrinsic apoptotic pathways were triggered by such treatment. CK2 blockade coupled with chemotherapeutics resulted in an additive cytotoxic effect. Basal and TNFα dependent IκB degradation, as well as NFκB transcriptional activity upon TNFα stimulation were impaired by CK2 blockade in MM cells. A partial nuclear co-localization of the catalytic a subunit of CK2 and p50/p105 was observed by confocal microscopy in MM cells. Moreover, endogenous p50/p105 and CK2 could be immunoprecipitated in MM cell lines. We conclude that CK2 is a kinase pivotal for survival and proliferation of MM cells and its selective blockade is strongly cytotoxic to malignant plasma cells. CK2 regulates IκB protein levels and NFκB transcriptional activity, this latter effect being possibly mediated through physical association with NFκB transcription factors. Our findings suggest that the CK2 inhibition could be exploited as a novel therapeutic approach for MM.

PO-067
N-RAS AND K-RAS MUTATIONAL ANALYSIS IN NEWLY DIAGNOSED MULTIPLE MYELOMA PATIENTS: EVIDENCE FOR TWO NOVEL ACTIVATING MUTATION

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The RAS family members are among the most commonly mutated oncogenes in multiple myeloma (MM). Activating mutations of N-RAS and K-RAS have been demonstrated to result in growth factor independence and suppression of apoptosis of MM plasma cells. Nevertheless, the incidence and prognostic significance of RAS gene mutations in MM has to date been reported with some discrepancies. This issue has now gained new interest since the recent development of novel anticancer drugs (such as the farnesyl transferase inhibitors) acting by blocking oncogenic RAS-signaling pathway, which could be successfully exploited for the therapy of a subset of MM patients. We investigated RAS gene mutations in 85 newly diagnosed MM patients, who were randomized to receive either a single or double autologous peripheral blood stem cell (PBSC) auto-transplant(s), following remission induction chemotherapy with VAD and high-dose cyclophosphamide. For this purpose, genomic DNA obtained from bone marrow samples was analyzed by primer-specific amplification of N- and K-RAS exons 1 and 2, followed by direct automatic sequencing. We detected a total of 31 point mutations in 30 out of 77 (39%) evaluable patients. Nine mutations were found in N-RAS: one at codon 12, two at codon 13 and six at codon 61. Twenty-two mutations were found in K-RAS: eight at codon 12, four at codon 13, five at codon 16, two at codon 31, two at codon 61. One patient showed evidence of two distinct K-RAS mutations (both at codon 13 and at codon 61). To our knowledge, this is the first time that K-RAS mutations at codons 16 (AAG to AAC) and 31 (GAA to CAA) are reported in MM. To date, such mutations have been found only in adrenocortical tumors and have recently been demonstrated as activating. No significant association was observed between any RAS mutation and age, gender, bone marrow infiltration, stage of disease, immunoglobulin isotype, creatinine, C-reactive protein and β2-microglobulin levels. As far as response to treatment was concerned, no major differences emerged between patients with and without a mutated RAS gene. However, patients who showed mutations affecting K-RAS codons 12 and 13 (n=12) had a significantly shorter event-free survival (EFS; median, 21 vs. 32 months; p = 0.03) with respect to patients who did not (n=65). It is concluded that: a) RAS activating mutations are a frequent event (39%) in newly diagnosed MM patients; b) in our series of patients treated with high-dose chemotherapy and single or double PBSC autotransplant(s), K-RAS mutations at codons 12-13 are associated to a worse outcome in terms of EFS, thus confirming the hypothesis that different RAS gene mutations cause a different degree of activation of the downstream effector pathways. Analysis of a larger series of patients is required to consider the impact on clinical outcome of the previously unreported K-RAS mutations at codon 16 and 31.

Funding: This work was supported by University of Bologna, Progetti di Ricerca ex-60% (M. C.); by MIUR and FIRB projects; by COFIN 2003, by the Italian Association for Cancer Research (AIRC), by the Fondazione del Monte di Bologna e Ravenna and by AIL.

PO-068
RANK/RANKL EXPRESSION IN MULTIPLE MYELOMA BONE MARROW ENVIRONMENT AND ITS ROLE IN IL-6 AND IL-11 UP-REGULATION

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The receptor activator of NF-κB ligand (RANKL) has a critical role in osteoclast activation. Recently, it has been demonstrated that human multiple myeloma (MM) cells up-regulate RANKL in bone marrow stromal cells (BMSC). To further investigate the role of RANKL in the pathophysiology of MM, we have evaluated the expression of RANKL and its receptor
CHEMOKINE RECEPTORS CXCR3 AND CXCR4 EXPRESSION AND ROLE IN PLASMA CELL MALIGNANCY

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Chemokine receptors, CXCR3 and CXCR4, are expressed on normal B and plasma cells being involved in regulation of chemotaxis and migration. In this study we have evaluated the potential expression and role CXCR3 and CXCR4 on human myeloma cells. First we found that fresh purified CD138+ plasma cells obtained from 25 multiple myeloma (MM) patients at the diagnosis expressed both CXCR3 and CXCR4 on the membrane in a wide range of expression. A significant increase of both chemokine receptors was found in comparison with normal subjects, moreover a correlation between CXCR3 expression and the clinical stage of MM patients was found. Human myeloma cell lines (HMC-Ls), RPMI-8226, OPM-2, XG-1, XG-6, OPM-2 established from patients with plasma cell leukemia, also expressed CXCR4 at high intensity. CXCR3 was expressed in 2 (RPMI-8226 and OPM-2) out of 5 HMC-L tested at lower intensity. CXCR3 expression was cell cycle dependent being associated with the proliferative phase of cell cycle. In addition CXCR3 expression on HMC-L detected by both flow cytometry and immunoistochemistry, was up-regulated during cell apoptosis induced with CD95 stimulation or IL-6 deprivation. Using an ELISA test we have also demonstrated that 3 out 4 HMCL produced the CXCR3 ligand and IFNgamma inducible protein (IP-10). A significant inhibitory effect on HMCL apoptosis was observed by treating them with IP-10 at concentration ranging between 50 to 100 ng/ml. In conclusion our data indicate that myeloma cells express CXCR3 and CXCR4 with a different pattern and at higher intensity as compared to normal subjects and that CXCR3 expression by myeloma cell is correlated with cycle and apoptosis and in turn CXCR3 activation by IP-10 can affect myeloma cell survival.

HUMAN MYELOMA CELLS INHIBIT OSTEOBLAST FORMATION AND DIFFERENTIATION IN THE BONE MARROW ENVIRONMENT

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Multiple myeloma (MM) is a plasma cell malignancy characterized by the high capacity to induce osteolytic lesions. The histomorphometric studies, performed in MM patients, have demonstrated that MM patients with high plasma cell infiltrate or active disease are characterized by a lower number of osteoblasts and a decreased bone formation that contributes, together with the increased osteoclast activity, in the development of bone lesions. However the mechanisms by which myeloma cells reduce bone formation are not completely identified. In this study first we have investigated the effect of human myeloma cell lines (HMC-Ls) on proliferation and survival of osteoblast–like cell lines MG–63 and primary human osteoblast cells (hOB) in a co-culture system. We found that conditioned medium (CM) of RPMI-8226, U266, XG-1 and XG-6 significantly reduced the number osteoblastic cells and suppressed osteoblast proliferation of both MG–63 and hOB. HMC-Ls are able to significantly induce apoptosis of human osteoblastic cells either in presence or absence of a transwell insert even if the cell–to–cell contact condition was more effective. CD95/FAS+ osteoblastic cells, as MG–63, are more sensitive to HMC-Ls apoptosis. Consistently the presence of blocking anti-FAS ligand Ab in the co-culture reduced the pro-apoptotic effect but not completely. Similarly we found that blocking anti-TRIAL Ab also reduced osteoblast apoptosis but did not completely blunt the pro-apoptotic effect of TRIAL positive HMC-Ls. Further, we have investigated the effect of HMC-Ls on the formation of osteoblast progenitors in long-term human bone marrow (BM) cultures. In this system we found that HMC-Ls significantly inhibited both the number of the Colony Forming Unit–fibroblast (CFU-F) after 15 days and of the CFU-OB after 21 days either in presence or absence of a transwell system even if the cell–to–cell contact induced a more potent inhibitory effect. Moreover, in a co-culture system, we found that myeloma cells inhibited the osteoblast dif-
ferentiation by BM stromal cells inhibiting the osteoblast marker osteocalcin and collagen I. In conclusion our data indicate that human myeloma cells inhibit osteoblast proliferation, induce osteoblast apoptosis and decrease human osteoblastogenesis. These effects could be responsible of the decreased bone formation observed in MM patients.

**PO-071**

**EXPRESSION OF OSTOPONTIN BY HUMAN MYELOMA CELLS AND ITS INVOLVEMENT IN MYELOMA INDUCED ANGIOGENESIS**

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Osteopontin (OPN) is a multifunctional bone matrix glycoprotein that interacts with CD44 as major receptor. OPN has been shown to be involved in angiogenesis, cell survival and tumor progression. In this study we have investigated the production and the potential role of OPN in multiple myeloma (MM). First, we found that human myeloma cells expressed OPN mRNA and its regulating gene the core-binding factor (CBFα1). OPN protein production and release by myeloma cells have been also demonstrated by westernblot analysis and ELISA assay, respectively. Moreover we found that IL-6 and IGF-I induced an increase of OPN production by myeloma cells and in turn OPN stimulated myeloma cell proliferation. In an in vitro angiogenesis system we show that OPN production by myeloma cells contribute to the pro-angiogenic effect of myeloma cells. OPN production was investigated in 52 newly diagnosed MM patients stage I-III. In this cohort of patients we found that purified bone marrow (BM) CD138+ MM cells from 20 out of 52 patients expressed OPN. Higher OPN protein levels were detected in BM plasma of MM patients in comparison to control subjects, moreover BM angiogenesis was significant higher in MM patients positive for OPN as compared to those negative. In conclusion our data highlight that OPN is directly produced by human myeloma cells and detected in a subset of MM patients with higher angiogenesis suggesting a potential involvement of OPN in the pathophysiology of MM.

**PO-072**

**VASCULOGENESIS IN PATIENTS WITH MULTIPLE MYELOMA THROUGH BONE MARROW MACROPHAGES**


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Tumor neovascularization forms through angiogenesis (sprouting from existing vessels) and vasculo-ogenesis (sprouting from precursor mesenchymal cells). In embryo, cells of this type cluster and differentiate into hematopoietic stem cells and at periphery into endothelial precursor cells (or angioblasts) which ultimately give rise to vessels. In post-natal life, angioblasts exist in peripheral blood and tissues, where differentiate into vessels under angiogenic stimuli, including VEGF and bFGF produced by ischemic or tumor cells. Angiogenesis is important for progression of multiple myeloma (MM). It is also important as a prognostic factor. Plasma cells secrete growth and differentiation factors for endothelial cells (EC), such as VEGF, bFGF, hepatocyte growth factor and angiopoietins, which are indeed highly found in the bone marrow than peripheral blood. Vasculo-genesis in MM, however, is still under scrutiny. Here we investigated some aspects of vasculogenesis in MM generated from bone marrow macrophages. These cells were isolated from heparinized aspirates with anti-CD14-conjugated microbeads, and left to differentiate into endothelial cells upon exposure to VEGF and bFGF. Macrophages were stimulated daily for one week with VEGF at a 50 ng/ml medium and/or bFGF at 10 ng/ml. Stimulated macrophages were studied by immunocytochemistry, western blot and RT-PCR for their phenotype and angiogenic capacity in the in vitro Matrigel assay. As a control, the THP-1 macrophage cell line (American Type Culture Collection) differentiated by exposure to PMA for 72 hours was used. By using RT-PCR, resting macrophages showed expression of CD14, while not that of FVIII-related antigen (FVIII-RA) and CD34. By using western blot and immunolocalization, we showed that stimulated macrophages developed a typical endothelial cell phenotype, expressing FVIII-RA, VE-cadherin, Tie-2/Tek and, to a lesser extent, VEGFR-2. They lost the CD68 molecule. The CD34 control molecule was absent in the THP-1 cells as well as in both resting and stimulated macrophages. After a 24-hour culture on matrigel and exposure to VEGF and bFGF, macrophages formed a closely-knit capillary plexus with multicentric junctions, similar to that produced by MM endothelial cells. To sum up, these data suggest that bone marrow macrophages of MM patients can transform into endothelial cells under an angiogenic stimulus and contribute to MM vascularization via mechanisms of post-natal vasculogenesis. Results also confirm that angiogenic cytokines may represent a target for therapeutic management of MM.
B cells may undergo sequential rearrangements at the light chain loci, despite already expressing light chain receptors. This phenomenon, known as secondary rearrangement, may occur during differentiation in the bone marrow (receptor editing) and in the periphery, at some point of the germinal center reaction (receptor revision). To study light chain recombinations that preceded the development of a marrow plasma cell we used multiple myeloma as a single cell-model and, taking advantage of the fact that Ig light chains usually rearrange before Ig ones, we used PCR to analyze the Ig locus of twenty-nine Ig myeloma cases. The results indicated that all Ig alleles were inactivated via rearrangement of the \( \kappa \) deleting element, more frequently to a V segment (69%) than to the intronic recombination signal sequence (31%). Eighteen alleles (16 myeloma clones) had previous V-J attempts, and these revealed increased utilization of distal V and J gene segments (J 56%), a marker of multiple sequential rearrangement. In-frame V-J rearrangements were found in approximately 1/3 of available joints (5/18, with one involving a V pseudogene), each one in different myeloma clones: 3 were identical to germline (i.e. compatible either with editing in the bone marrow or with revision before the onset of somatic changes), while 1 had several nucleotide substitutions indicating inactivation after the germinal center reaction had been initiated. The present findings have relevance for light chain genetics and support the view that developing B-cells may undergo both early and late recombinations at the light chain locus. Sustained activity of recombination-activating genes may contribute to immunoglobulin translocations in B-cell neoplasias.

Multiple myeloma (MM) is a neoplastic proliferation of plasma cells characterized by a marked biological and clinical heterogeneity. Dysregulation of distinct putative oncopgenes, as a result of chromosomal translocations involving the IGH locus at 14q32, occurs frequently in MM. Such oncogenic events are thought to be linked with the transformation and clonal evolution of malignant plasma cells and may have a significance in the definition of different entities of the disease. In the present study, the expression profiles of FGFR3/MMSET, CCND1, CCND3, MAF and MAFB, involved respectively in the t(4;14)(p16.3;q32), t(11;14)(q13;q32), t(6;14)(p21;q32), t(16;14)(q23;q32), and t(14;20)(q32;q12), were investigated on purified plasma cell populations from 39 MM and 6 plasma cell leukemia (PCL) patients by DNA microarray analysis and compared with the presence of translocations as assessed by dual-color FISH or RT-PCR. The t(4;14) was found in 6 MM patients, the t(11;14) in 10 patients (9 MM and 1 PCL), the t(6;14) in one MM case, the t(14;16) in three cases (2 MM and 1 PCL), and the t(14;20) in one PCL. Translocations were associated with spiked expression of target genes in all cases. In addition, gene expression profiling allowed to the identification of putative chromosomal translocations dysregulating the CCND1 (1 MM and 1 PCL) and MAFB (1 MM and 1 PCL) without any apparent involvement of immunoglobulin loci. Notably, all of the translocations were found to be mutually exclusive. Interestingly, marked increased levels of MMSET expression have been found in one MM case in which a 4p16.3 allele was localized on an unidentified chromosome. Overall, our data support the notion that translocations (either or not involving the IGH locus) represent the mechanism of dysregulation of putative oncopgenes primarily involved in myelomagenesis and suggest the importance of combined molecular cytogenetics and gene expression approaches for the detection of genetic aberrations in MM.
PO-075
NONMYELOABLATIVE ALLOGRAFTING FOLLOWING AUTOGRAFFING IS FEASIBLE AND HAS A STRONG ANTI MULTIPLE MYELOMA ACTIVITY

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Background: The development of reduced intensity conditioning regimens (RIC) has renewed interest in allografting for patients with multiple myeloma (MM). Taking advantage of this new approach, we postulated that combining maximal tumor reduction achieved with autografting and the benefits of RICT, we could achieve more cures of multiple myeloma (MM) with acceptable toxicity. Design and Methods: Sixteen patients, 51 years of age (range, 36–63) with previously treated stage III MM were given melphalan 140 mg/m2 and autologous peripheral blood progenitor cells (PBPC) reinfusion. The regimen-related toxicities were moderate with a median of 8 and 11 days of neutropenia and thrombocytopenia, respectively. Forty-six to 156 days later (median, 79 days), the patients received fludarabine plus 2 Gy TBI and HLA-identical donor mobilized PBPC. Postgrafting immunosuppression consisted of cyclosporine (CSP) and mycophenolate mofetil (MMF). Engraftment occurred in 14 patients (87%). Results: Thus far, 10 patients (62%) are alive with 9 of them in continuous complete remission 11–36 months (median, 30 months) after transplants. Grade II–III acute GVHD occurred in 7 patients (43%) but no patient died of aGVHD. Three patients (18%) developed extensive chronic GVHD requiring intensive therapy. Six patients died; five of them of progressive disease and one of progressive disease combined with extensive cGVHD and interstitial pneumonitis. Conclusions: This 2-step approach is feasible and demonstrated to have a strong antiyeloma activity with reduced deaths due to acute toxicities.

PO-076
POLYMORPHISMS OF METHYLENETERAHYDROFOLATE REDUCTASE (MTHFR) AND SUSCEPTIBILITY TO MULTIPLE MYELOMA


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MTHFR reduces methylenetetrahydrofolate to methyltetrahydrofolate which is the main methyl donor for homocysteine remethylation to methionine and for DNA methylation. Two MTHFR polymorphism have been recently described 677C to T and 1298A to C. These polymorphism are associated with reduced activity of the enzyme promoting a genomic hypomethylation which would result in a reduced risk of carcinogenesis. Moreover the reduced function leads to the increase of 5,10 methylenetetrahydrofolate which increases the amount of thymidilate available for the DNA synthesis. A relationship between these polymorphisms and the susceptibility to colorectal cancer and acute leukemia has been recently reported demonstrating that individuals with variant allele (677T and 1298C) have lower susceptibility to these cancers than individuals with the wild type variants (677C and 1298A). Aims and Methods: In the present study we examined the prevalence of both MTHFR polymorphism among 100 multiple myeloma (MM) patients and 100 MM age and sex matched healthy controls. Patients characteristics were: M/F 55/45, median age at diagnosis 62y (range 34–87). Of these patients 10 were in IA stage, 36 in IIA, 2 in IIB, 50 in IIIA and 2 in IIIB stage. Seventeen patients had a prior history of monoclonal gamopathies of uncertain significance (MGUS). Fifty patients received standard chemotherapy (melphalan with prednisone or dexamethasone), 35 chemotherapy followed by autologous/allogeneic peripheral blood stem cell transplantation, 1 patient radiotherapy alone and 14 were untreated. Ninety-two patients are alive while 8 patients died after a median time of 51 months (range 12–83). The median follow-up was 24 months (range 1–216). Nineteen patients obtained complete response to chemotherapy, 39 partial response, 19 nineteen, 15 were not evaluable. The control population consisted of 100 individuals without cancer history. Results: Among the 100 patients the frequency of 677 wild type (CC) was 31% compared to 36% in controls. The heterozygous mutants genotype (CT) was detected in 44% of MM patients vs 45% in controls, while the homozygous mutants (TT) had a frequency of 25% vs 19% in patients vs controls. The frequency of 1298 wild type (AA) was 48% in patients group compared to 50% in controls. The heterozygous mutants genotype (AC) was detected in 44% of MM patients vs 37% in controls, while the homozygous mutants (CC) had a frequency of 8% vs 13% in patients vs controls. In the group with a prior history of MGUS for the C677T polymorphism 52.9% of patients were normal, 41% were heterozygous and 5.8% were homozygous and regarding polymorphism A1298C
35.2% of patients were normal, 52.9% were heterozygous and 11.7% were homozygous. Conclusions: MTHFR reduces methyleneTHF to MethylTHF, which is the main methyl donor for homocysteine remethylation to methionine and thus DNA methylation. On the other hand the substrate methyleneTHF acts as cofactor of thymidilate synthase necessary for DNA synthesis. The two polymorphisms 677CT and 1298AC are associated with a reduced activity of the enzyme. Previous studies have described a lower risk of colon cancer and acute lymphoblastic leukemia. In contrast to these observations we described a lack of correlation between these polymorphisms and the susceptibility to ALL in the Italian population. In the present study we report an analysis regarding the association between the susceptibility to MM and polymorphisms in the gene encoding the enzyme MTHFR. Statistical analysis did not disclose any significant association between MTHFR polymorphisms and MM but in the subgroup of patients with a prior history of MGUS we found a lower prevalence of the 677TT genotype (5.8% vs 25% in the myeloma group, p=0.043). This data could suggest that patients with MGUS and 677TT polymorphism develop less likely in MM. Obviously the small sample size imposes some cautions in fact further studies are necessary to confirm the influence of the MTHFR and the role of folate in myeloma pathogenesis.

PO-077
GENE EXPRESSION PROFILING OF HUMAN MYELOMA CELL LINES
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Multiple Myeloma (MM) is a neoplastic disorder of bone marrow plasma cells (PCs) characterized by a marked genetic heterogeneity. In particular, it has been demonstrated that MM PCs show complex chromosomal aberrations, the most frequent of which are chromosomal translocations involving the immunoglobulin heavy-chain locus (IGH) and a promiscuous array of partner loci. The result of these genomic alterations is the deregulation and overexpression of the target genes as a consequence of their juxtaposition to regulatory sequences of the IGH locus. The best characterised IGH translocations include t(11;14), t(4;14), t(14;16) and t(6;14), which respectively lead to the deregulated expression of the putative oncogenes CCND1, FGFR3/MMSET, MAF and CCND3 and have been identified in the majority of MM cases and human myeloma cell lines (HMCLs). To investigate the functional significance of different genetic lesions and the mechanism of action of different target genes we used Affymetrix DNA microarrays to analyse the gene expression profiles of a panel of 20 HMCLs that have been characterised for the more frequent IGH translocations. In particular, the panel included 8 HMCLs positive for t(4;14), 5 positive for t(14;16); 3 for t(11;14), 1 for t(6;14) and 5 negative for any known IGH translocation. The unsupervised analysis showed that samples clustering was only partially driven by the presence of the main chromosomal translocations. However, supervised analysis performed on HMCLs groups homogeneous for distinct types of IGH translocations, identified specific gene expression profiles associated with each of the deregulated genes. Furthermore, supervised analysis performed on HMCLs negative for any known IGH translocation identified specific up-regulation of a set of cancer germ line-specific antigens in these samples. Our analysis provides insights into the molecular pathogenesis of MM by identifying a number of genes specifically modulated as a result of different translocations. Given the evidence that distinct types of chromosomal translocations may be associated to different clinical and prognostic outcome, the validation of these findings on MM patients will be discussed.

PO-078
EVALUATION OF BONE RESORPTION MARKERS IN NEWLY DIAGNOSED MULTIPLE MYELOMA PATIENTS TREATED WITH THALIDOMIDE + DEXAMETHASONE AND ZOLEDRONIC ACID
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Bone involvement is frequently observed in multiple myeloma (MM) patients both at diagnosis and during the course of the disease. Evaluation of biochemical markers of bone turnover could offer a dynamic perspective of the effects of a given therapy on bone metabolism. In patients who were enrolled in the Bologna 2002 phase II clinical trial and treated at our center, markers of bone resorption (urinary NTX, PYR and DPYR and serum crosslaps) and bone formation (bone alkaline phosphatase-BAP and osteocalcin) were routinely evaluated at diagnosis and at various time points during therapy. By study design, all patients received four months of combined thalidomide (100mg/d for two weeks and 200 mg/d thereafter) and dexamethasone (40 mg/d, on d 1-4,
9-12, 17-20/28 d on odd cycles and on d 1-4/28 d on even cycles) therapy (THAL-DEX) as induction of remission before peripheral blood stem cell (PBSC) collection with high-dose cyclophosphamide and subsequent double autotransplants upon treatment with melphalan 200 mg/m². Zoledronic acid (ZOLE acid) was administered at 4 mg/28 d for at least 9 months. Data from 35 patients (19M, 16F, median age = 53 years) who underwent the first treatment phase have been collected so far. After 4 months of therapy with THAL-DEX and ZOLE acid a significant decrease in mean urinary NTX (57.1 ± 7.2SE nmol/mmol crea vs 20.45±3.3SE, p=0.000) and serum crosslaps (5786±846SE pmol/L vs 2108±324SE, p=0.000) was observed. In patients who responded favorably to THAL-DEX (n = 25), a significant reduction in both serum crosslaps (77%) and urinary NTX (72%) was observed, while in refractory patients (n = 10) no significant change was detected (30% decrease in urinary NTX and 29% reduction in serum crosslaps). A significant decrease in bone formation markers was observed in both sensitive and refractory patients, this can be attributed to DEX therapy; however, this finding needs to be confirmed at a subsequent analysis performed at the end of the whole treatment program. It is concluded that among all the markers of bone turnover, serum crosslaps and urinary NTX are the ones most strictly related to actual bone resorption; combined THAL-DEX and ZOLE acid administered as primary therapy for patients with newly diagnosed and symptomatic MM seem to be highly effective in reducing bone resorption, although the relative contribution of each of these drugs cannot yet be determined.

To evaluate the feasibility and efficacy of Thal in combination with Dexamethasone (Dex) and cyclophosphamide (CTX) for relapsed/refractory multiple myeloma (MM). Between October 2001 and April 2004, 30 patients (pts) (20 M/10 F) with relapsed/refractory MM were enrolled in an open-label trial of oral low dose Thal (100–200 mg/day) plus Dex(40 mg, day 1–4, every month) and CTX (500 mg iv/week). Main pre-treatment characteristics were the following: median age 68 years (range 42–79); median B2M 6.25 mg/L (range 1.2–11.6); median bone marrow plasma cell infiltration 60% (range 4–100) and median time from MM diagnosis to treatment was 41 months (range 6–132). All pts were heavily pre-treated: in particular, 19 pts received a median of 3 pre-treatment chemotherapy regimens (range 1–5) and 11 underwent autologous stem cell transplantation. In addition, 20 pts showed disease progression during previous treatment with Thal alone or combined with Dex. With a median follow-up of 12 months (1–24), all 30 pts were evaluable for response. According to the EBMT/IBMTR/ABMTR criteria, 23 (66.6%) showed a degree of response, including 2 cases who had a complete remission, 15 a partial response and 6 a minimal response; 4 pts (23.4%) showed no change and 3 (10%) a progressive disease. At present, 11 pts are alive and 8 are still maintaining the response from a median of 12 months (1–24), with a 30% EFS and 30% OS at 2ys. According to WHO criteria, adverse effects were moderate (grade <=2). No cases of thrombocytopenia grade =>2 were observed, while 2 pts experienced neutropenia requiring supportive treatment with G-CSF. Other side effects included grade <=2 constipation (30%), somnolence (35%) or dizziness (10%). No cases of deep venous thrombosis were observed. These results show that the Thal plus Dex and CTX is an active and feasible combination therapy in heavily pre-treated multiple myeloma pts, including those relapsing after Thal-Dex therapy.

**PO-079**

**THALIDOMIDE IN COMBINATION WITH DEXAMETHASONE AND CYCLOPHOSPHAMIDE FOR RELAPSED/REFRACTORY MULTIPLE MYELOMA**

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The anti-myeloma effect of thalidomide (Thal) has been demonstrated in several clinical trials. Recent data have also indicated that Thal can increase the therapeutic effect of chemotherapy and might be able to overcome drug resistance. Response rates of 25% with Thal used as a single agent, and up to 75%, when used in combination with other agents, have been observed. The optimal schedule, dosage and association with other drugs is still not established.

**PO-080**

**ENDOTHELIAL DIFFERENTIATION OF HEMATOPOIETIC STEM CELLS FROM PATIENTS WITH MULTIPLE MYELOMA**


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Vasculogenesis is a physiological process typical of fetal development. New blood vessels develop from...
undifferentiated precursors (or angioblasts), different from angiogenesis in which vessels origin from existing ones. Several studies have shown the presence of circulating and tissue angioblasts also in post-natal life. These cells are able to differentiate into endothelial cells and other several histotypes under specific stimuli. There are some evidences that vasculogenesis also takes place in post-natal life in ischemic tissue and tumors. In tumors, near angiogenesis, vasculogenesis contributes to the formation of the microvascular plexus that is important for local and systemic diffusion. Here, we show that hematopoietic stem cells of multiple myeloma (MM) patients, purified with an anti-CD133 antibody from peripheral blood after mobilization with cyclophosphamide and G-CSF, are able to differentiate into cells with endothelial phenotype after about 20 days culture upon exposure to VEGF and bFGF. Stem cells were seeded in 75 cm² flasks coated with fibronectin in IMDM supplemented with 10% fetal bovine serum (FBS), 10% horse serum, 10⁻⁶ M hydrocortisone, 50 ng/mL VEGF and 10 ng/mL bFGF. During the culture, stem cells gradually changed their phenotype, lost the typical antigen of undifferentiated stem cells (CD133), and acquired a mature endothelial cell phenotype. By using western blot and RT-PCR we demonstrated that this phenotype is characterized by a high expression of VEGF receptor-2 (VEGFR-2/KDR), factor-VIII-related antigen (FVIII-RA) and VE-cadherin. By using immunofluorescence, we showed that this differentiation process went on slowly in step with the culture. Actually, parallel to the endothelial antigen expression, cells adhered to the fibronectin, spread and acquired the typical endothelial cell shape. Finally, after about 20 days culture, differentiated cells were able to form a capillary network on Matrigel (capillarogenesis) upon a 24-hour exposure to both VEGF and bFGF. In conclusion, our data suggest that angiogenic VEGF and bFGF released by MM plasma cells may induce the differentiation of hematopoietic CD133+ stem cells into endothelial cells that, near angiogenesis, contribute to the development of a tumoral vascular tree, thus promoting MM progression and diffusion. These data confirm the usefulness of antiangiogenic drugs, particularly the VEGF and bFGF inhibitors, in the therapeutic management of MM.

High dose cyclophosphamide (HD-CTX) is the most commonly used mobilizing regimen for patient with multiple myeloma (MM). However, this regimen results in substantial hematologic and extrahematological toxicity, often needing hospitalisation. In addition, the time to collection is variable. Different attempts have been made in order to investigate the efficacy and toxicity of alternative approaches aiming at collection of peripheral blood stem cells (PBSC) with reduction of side effects and costs of the procedure. Accordingly, we adopted a combination regimen based on the combination of vinorelbine (VNB) and CTX. From July 2003 to April 2004, 25 consecutive patients with MM received VNB 30 mg/m² intravenously (iv) on day 1 and CTX 1500 mg/m²/iv on day 2, followed by G-CSF 10 µg/Kg/ from day 3 to CD34 positive (CD34+) cells peak, all in an outpatient setting. The median age of patients was 57 (range 37-73) and the stage at diagnosis was II in all cases. In 19 patients VNB-CTX was given as part of a first line therapy after 3 courses of VAD with (n=6) or without (n=13) thalidomide. In 6 patients (25%) the VNB/CTX mobilization regimen was given after a second or further line of chemotherapy; of note, among these 4 patient had been previously autografted. Collection was successful in 19/19 (100%) untreated patient and in 3 out of 6 (50%) pretreated cases, 1 of which had been previously autografted. The peak value of CD34+ cells was observed after a median of 9 days (range 8-11). Overall, following mobilization a median of 8.7 CD34+ cells (range 2.2-17.4) were collected after a median of two apheresic procedures (range 1-2). Of note, the median number of CD34+ cells collected was of 8.8 (1.22-15.36) for untreated patients as compared to 4.4 (4.0-17.4) for pretreated. There were no episodes of grade 3-4 neutropenia or thrombocytopenia, while one patient required one packed rbc unit. Fever or hemorrhage did never occur, and there was no episode of extrahematologic toxicity. Among 22 mobilizing patients, 18 did actually receive autologous stem cell transplantation, while 4 are waiting for the procedure. Hematopoietic recovery in autografted patients was fast with a median time to neutrophil and platelet recovery of 12 and 14 days, respectively. We conclude that VNB/CTX regimen results in highly efficient mobilization of CD34+ cell in patients with MM and represents an appealing alternative to HD-CTX therapy. Major advantages include predictable time for collection, negligible toxicity, feasibility on an outpatient basis, and consequent reduction of costs. Data on pretreated patients need to be confirmed on larger series.

PO-081

VINORELBINE, CYCLOPHOSPHAMIDE AND G-CSF AS MOBILIZA-
TION REGIMEN FOR PATIENTS WITH MULTIPLE MYELOMA

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CURRENT ISSUES IN TOTAL BODY SCINTIGRAPHY WITH SESTAMIBI IN MULTIPLE MYELOMA

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Total body scintigraphy with Technetium-99m Sestamibi (Mibi) has been used for many years to evaluate the bone lesions of patients with Multiple Myeloma (MM) and since the early trials it has been compared with skeletal radiology. We have evaluated 58 patients with MM. Mibi scintigraphy was positive (score >2) in 44 patients, with a pattern of uptake diffuse (D) in 32, focal (F) in 2, diffuse + focal (D+F) in 10. The score correlated with stage, plasma cells invasion and monoclonal protein. Mibi scintigraphy was positive more often than skeletal X-ray, but not all the bone lesions showed by X-Ray correspond to zones of higher Mibi uptake. Comparison to Magnetic Resonance Imaging (MRI) showed that a diffuse uptake pattern did not always correspond to osteolytic areas. This is due to the fact that Mibi scintigraphy is based on cell metabolism whereas the other imaging methods account for structural lesions. Moreover, Mibi did not prove effective in identifying small lesions (<1 cm), especially of the skull: it picked-up only 4 out of 12 patients affected by osteolytic skull lesions. It is too early to draw conclusions on these preliminary results.

BORTEZOMIB (VELCADE) AS SALVAGE THERAPY FOR ADVANCED MULTIPLE MYELOMA: A MULTICENTRE SURVEY OF ITALIAN PATIENTS TREATED OUTSIDE OF CLINICAL TRIALS

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Bortezomib (VELCADE, formerly PS-341, Millennium) is a novel first-class agent that inhibits the proteasome, a multicatalytic cellular enzyme whose activity entails several molecular mechanisms, including, in particular, the NF-kB pathway. Recent phase I-II clinical trials have demonstrated the efficacy
was given at the dose of 1.3 mg/m² body surface area and 18 patients bortezomib was also received thalidomide. One patient also received dexamethasone and one patient dexamethasone plus thalidomide. A reduced dose of 0.8 mg/m² was administered in seven subjects receiving concomitantly liposomal-doxorubicin (12 mg/m²) and dexamethasone (20 mg for 4 days) every cycle. A total number of 60 cycles was administered (range 0.5–5 per patient). Two patients with very advanced disease and relevant co-morbidities died of cardiac failure and cerebral hemorrhage, respectively, after the first two doses. Three additional patients died of progressive disease during or shortly after bortezomib treatment. Grade 3–4 WHO hematological toxicity (mainly thrombocytopenia) occurred in four patients, determining the need of reducing or temporarily stopping the treatment. Fungal pneumonia (candida) was observed in a patient receiving combined therapy. Four patients experienced minor infections. In another patient a cutaneous leucocytoclastic vasculitis (diagnosed by skin biopsy) developed under bortezomib therapy. Diarrhoea, constipation, somnolence, nausea, vomiting, fever, bone pain and mild neurological symptoms were observed in eleven patients. So far, fourteen patients are evaluable for response. According to Bladé criteria, one patient achieved complete remission (CR), which lasted two months, nine patients obtained partial remission (PR), four patients evidenced stable (SD) or progressive (PD) disease. Six patients with PR maintain their response after 2–9 months. In one of them, relapsed after double autologous stem cell transplantation, a program of unrelated, non-myeloablative stem cell transplantation could be started after response to bortezomib. In 3 patients with PR the disease relapsed after 4–6 months. Responding patients also had evidence of improved hemoglobin values, performance status, quality-of life and levels of non-involved immunoglobulins. As of May 30, 2004, 17 patients are alive and 12 out of them are still on bortezomib therapy. Updated results will be presented at the VIII Congress of the Italian Society of Experimental Hematology, Pavia, September 14-16, 2004.

PO-084

IN VITRO GENERATION OF ANTIMYELOMA ACTIVITY BY ZOLEDRONIC ACID: ROLE OF EFFECTOR (CD45- CD27-) γδ T CELLS

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The role of innate effector cells such as macrophages, NK cells, NKT cells and γδ T cells in natural tumor immunity and tumor immunotherapy has recently been revisited. Circulating γδ T cells (1–5% of peripheral blood T cell) are naturally activated by aminobisphosphonates (NBPs), a new class of drugs commonly used in MM and other cancer patients to effectively prevent osteoclast activation and skeletal related events. The aim of this work was to investigate the immunomodulatory properties of zoledronic acid (Zol), the most potent NBP clinically available. We have investigated in 45 normal donors and 45 MM patients the in vitro reactivity of gammadelta T cells to Zol. Zol induces a rapid expansion (7 days) of gammadelta T cells in normal donors and MM patients in the presence of very low doses of IL-2. However, the mean total number of γδ T cells was lower in MM patients. This reflected the presence of 50% of MM patients whose γδ T cells did not proliferate to Zol [non-responders (NR)]. Unexpectedly, the antitumor activity generated by Zol against myeloma cell lines and primary myeloma cells was similar in NR and MM patients whose gammadelta T cells proliferated to Zol [responders (R)]. Depletion and blocking experiments showed that antitymoma activity was strictly dependent on gammadelta T cells in R and NR. Phenotyping showed that R and NR had distinct distribution in memory (CD45RA– CD27+) and effector (CD45RA– CD27-) γδ T cells. The former were the predominant subset in R, whereas the latter were the predominant subset in NR. Memory γδ T cells display high proliferative capacity and low effector function, whereas effector gammadelta T cells show the opposite pattern. This can explain why R and NR have different proliferative capacities, although they have the same capacity to generate antitumor activity. In conclu-
We found that at high Zol concentrations (1-4 M) evaluated the ability of Zol in affecting their expression. The central role of adhesion molecules in MM, we evaluated by flow cytometric detection of fluoresceine-labelled Annexin V, to be associated with increased cell apoptosis (p<0.002). In addition, staining of nuclei with nuclear fluorescent dyes highlighted morphological changes typical of apoptosis, i.e. chromatin condensation, nuclear fragmentation, and formation of dense rounded apoptotic bodies. Given the central role of adhesion molecules in MM, we evaluated the ability of Zol in affecting their expression. We found that at high Zol concentrations (10-4 M), VCAM-1, ICAM-1, VLA-4 and CD40 were reduced, while ICAM-3 and CD44 remained unmodified. To evaluate whether the reduction of adhesion molecules expression was correlated to a down-regulation of cytokine production, we exposed BMSCs to different Zol concentrations. The analysis of culture supernatants showed that myeloma BMSCs express IL-6, negligible levels of tumor necrosis factor-alfa, and no IL-1β. Short exposure to low concentrations of Zol was sufficient to cause a decrease of IL-6 production by BMSCs. In conclusion, these data demonstrate the great capacity of Zoledronic acid to interfere with myeloma microenvironment by inducing apoptosis, reducing cell proliferation and IL-6 production, and overall by modifying the pattern of expression of the adhesion molecules involved in myeloma cell binding to BMSCs.

**PO-085**

**EFFECTS OF ZOLEDRONIC ACID ON MARROW MICROENVIRONMENT IN MULTIPLE MYELOMA**


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In multiple myeloma (MM) plasma cells localize within the bone marrow interacting with the stromal cells through the expression of cell-surface adhesion molecules. These induce myeloma cells growth mediated by autocrine and paracrine production of several cytokines (IL-6, IL-1β, TNF-α, etc.). The aim of this study was to investigate the capacity of zoledronic acid (Zol) to interfere in vitro with bone marrow stromal cells (BMSCs) from patients with MM. The effect of Zol on BMSCs proliferation was analysed using MTT assay. After 3 days of treatment Zol caused a dose-dependent decrease of cell number (p<0.0001). This strong cytotoxic effect was demonstrated through flow cytometric detection of fluoresceine labelled Annexin V, to be associated with increased cell apoptosis (p<0.002). In addition, staining of nuclei with nuclear fluorescent dyes highlighted morphological changes typical of apoptosis, i.e. chromatin condensation, nuclear fragmentation, and formation of dense rounded apoptotic bodies. Given the central role of adhesion molecules in MM, we evaluated the ability of Zol in affecting their expression. We found that at high Zol concentrations (10-4 M) VCAM-1, ICAM-1, VLA-4 and CD40 were reduced, while ICAM-3 and CD44 remained unmodified. To evaluate whether the reduction of adhesion molecules expression was correlated to a down-regulation of cytokine production, we exposed BMSCs to different Zol concentrations. The analysis of culture supernatants showed that myeloma BMSCs express IL-6, negligible levels of tumor necrosis factor-alfa, and no IL-1β. Short exposure to low concentrations of Zol was sufficient to cause a decrease of IL-6 production by BMSCs. In conclusion, these data demonstrate the great capacity of Zoledronic acid to interfere with myeloma microenvironment by inducing apoptosis, reducing cell proliferation and IL-6 production, and overall by modifying the pattern of expression of the adhesion molecules involved in myeloma cell binding to BMSCs.

**PO-086**

**INCREASE OF SOLUBLE CD30 (SCD30) AND EOSINOPHIL COUNT IN MYELOMA PATIENTS TREATED WITH A REGIMEN INCLUDING THALIDOMIDE**


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Although treatment with thalidomide is becoming a standard therapy for resistant myeloma, the mechanism of action of this drug is not fully understood yet. Besides its anti-angiogenic effect, an immunomodulatory activity has also been postulated with a possible switch from a Th1 to a Th2 response. In this context we evaluated the concentration of soluble CD30 (that could indicate a Th2 type immune response) in serum of patients affected by multiple myeloma and treated with a combination of Thalidomide (200 mg/die continuously), Dexamethasone (40 mg/die for 4 days/month), and Cyclophosphamide (100 mg/die continuously). Twenty patients were included in the study. All of them were affected by relapsed or resistant multiple myeloma. Serum sCD30 concentration was measured before treatment and once a month for the first 4 months. At time of starting treatment sCD30 values were similar to normal control (median value 48 vs 63 U/ml, ns). After treatment, serum sCD30 concentration increased in every patient but one, starting from the first month post-treatment. sCD30 median value was 134 U/ml after 1st month (p<0.001), 112 U/ml after 2nd month (p<0.01), 120 U/ml after 3rd month (p<0.05) and 108 U/ml after 4th month (p<0.05). However, no correlation was found between the amount of sCD30 increase and response to treatment. In the attempt to evaluate the source of sCD30, in ten patients we measured the percentage of CD30+ T and B lymphocytes before and 1 month after treatment but we did not observe any difference. In addition, we did not find expression of CD30 in CD138 positive cells in the bone marrow of 5 patients examined before treatment. In this series we also observed an increase of eosinophil count that paralleled the increase of sCD30. Median eosinophil count before treatment was 69×10^9/L and it increased to 1.0×10^9/L after the 1st month (p=n.s.), 1.88×10^9/L after the 2nd month (p<0.05), 1.26×10^9/L after the 3rd month (p=n.s.) and 1.80×10^9/L after the 4th month (p=n.s.). This finding is intriguing because IL5 is another Th2 cytokine and it has also been demonstrated that purified human eosinophils express detectable levels of CD30 (Matsumoto, J Immunol 2004). However, we did not find any correlation between the eosinophil count and the sCD30 concentration. No correlation was also
present between the percentage of plasma cell bone marrow infiltration and the eosinophil count or the entity of its increase. Furthermore, no difference in the eosinophil count or in the magnitude of increase was observed among the different degree of response (from minimal to complete response). Finally, none of the thalidomide's side effects was correlated to the eosinophilia. In conclusion, our evaluation in patients treated with a combination of Thalidomide, Dexamethasone and Cyclophosphamide indicates that this treatment might work through stimulation of immune system that in turn could be responsible for the increase of sCD30 and eosinophils but the relationship between these modifications and the efficacy of the treatment remains to be clarified.

PO-087
USE OF PERCUTANEOUS KYPHOPLASTY FOR PAINFUL VERTEBRAL BODY FRACTURES IN PATIENTS WITH ONCO-HEMATOLOGICAL DISEASES

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Vertebral fractures are the most important source of morbidity in patients with multiple myeloma (MM) and one of the most disabling effect of a prolonged corticosteroid therapy for haematological patients (pts). Non-operative treatments such as cytotoxic drugs and radiotherapy (in patients with MM) or analgesic medications and bisphosphonates (in all patients) are increasingly used but any of these agents is effective in relieving pain in all cases. Surgical management generally involves vertebrectomy and reconstruction with PMMA (polimetiltmetacrylate) bone cement, but this technique is not suitable for the treatment of patients with multifocal vertebral lesions. Another treatment for vertebral fractures is the vertebroplasty that involves the percutaneous injection of PMMA into a fractured vertebral body; however this procedure does not reexpand a collapsed vertebra, it can just reinforce and stabilize fracture and it is often related to leakage of PMMA through cortical defects, with epidural compression of neural elements. A recent modification of vertebroplasty is the percutaneous balloon kyphoplasty (PBK): under general anesthesia, a 13-gauge needle is introduced trough a small dermatotomy and advanced to the posterior aspect of each pedicle along its superolateral cortex; the needle is directed, medially and caudally through the pedicle; then, using a hand-mounted drill, bilateral channels are created to reach the posterior tract of VB. Through the channel a high-pressure balloon is introduced and inflated to reduce the VB back to its original height; the cavity so obtained is subsequently filled with the PMMA. Balloon inflation and the PMMA filling were performed under fluoroscopy vision. Between March and December 2003 we underwent 7 patients to percutaneous kyphoplasty: 4 pts were affected by MM and 3 pts were affected by Lymphoma non Hodgkin, with vertebral fractures after corticosteroid therapy. In the group of pts with MM median age was 72 years (range 70-83), the average of vertebral fractures for patient was 2 (range 1- 4) and the pts with multiple fractures underwent 2 treatments; in this group, the pre-treatment Karnofsky performance status was 40 (range 60-30) and VAS-score (pain score with points assigned subjectively by the patients in a range 0, absence of pain, and 10, maximum pain) was 9 (range 8- 10), respectively; after treatment Karnofsky grade and VAS-score were 80 (range 50-90) and 2 (range 2-5), respectively. In the group of pts with lymphoma median age was 65 years (range 60-73) and they presented an isolated vertebral fracture; before treatment in the Karnofsky performance status grade was 50 (range 50-60) and VAS-score was 10; after treatment Karnofsky grade and VAS-score were 90 and 2, respectively. In conclusion, kyphoplasty was effective in relieving pain and improving life quality of the patients. Moreover in pts with MM the exothermic reaction of PMMA injected into the vertebral body may contribute to the effect of the procedure. The safety and efficacy of this procedure needs to be evaluated in a larger number of patients.

PO-088
DOWN-MODULATION OF ERK PROTEIN KINASE ACTIVITY INHIBITS VEGF SECRETION BY HUMAN MYELOMA CELLS

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The mitogen-activated protein (MAP) cascade leading to the activation of Extracellular signal-Regulated Kinases 1/2 (ERK1/2) is critical for regulating myeloma cell growth, however the relationship of ERK1/2 activity with Vascular Endothelial Growth Factor (VEGF) production and the effects of its down-modulation in myeloma cells are not elucidated. In this experimental study we found that the treatment with MAP/ERK Kinase 1 (MEK1) inhibitors PD98059 or PD184352 produced a reduction of phospho-ERK1/2.
(p-ERK1/2) levels in myeloma cells of more than 80% and prevented the increase of p-ERK1/2 induced by IL-6. MEK1 inhibitors also induced a significant inhibition of myeloma cell proliferation and blunted the stimulatory effect induced by IL-6. A significant inhibition of basal VEGF secretion by myeloma cells alone or in a co-culture system and the suppression of the stimulatory effect of IL-6 on VEGF has been observed by either PD98059 or PD184352. Moreover, we found that the PI3K kinase inhibitors but not p38 MAPK inhibitors reduced VEGF secretion by myeloma cells and increase the inhibitory effect of MEK1 inhibitors.

In an in vitro model of angiogenesis, we demonstrated that MEK1 inhibitors impair vessel formation induced by myeloma cells and restored by VEGF treatment, suggesting that the down-modulation of ERK1/2 activity reduces myeloma-induced angiogenesis by inhibiting VEGF secretion.

PO-089
T CELL-REGULATION OF OSTEOCLAST FORMATION AND SURVIVAL INVOLVING OPG/TRAIL INTERACTION IN AN IN VITRO MODEL FROM HUMAN MULTIPLE MYELOMA


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Multiple myeloma (MM) is a B-cell neoplasm characterized by clonal expansion of malignant plasma cells in bone marrow (BM), with frequent occurrence of lytic bone disease resulting from an enhanced bone resorption, related to increased osteoclast (OC) recruitment and activity and low bone formation. The interaction between MM cells and BM microenvironment is essential for maintenance and progression of the disease process. On the basis of the novel paradigm for T cells as regulators of bone turnover, we investigated the potential involvement of MM T cells and their expression of the major mediators of osteoclastogenesis. Therefore, we performed in vitro studies using unfractionated peripheral blood mononuclear cells (PBMCs) derived from 32 MM patients, and 32 subjects with nonneoplastic disease lacking any skeletal involvement as controls; parallel T cell-depleted PBMC cultures from the MM patients were established as well. In the unstimulated unfractionated PBMC cultures from the MM patients with lytic bone lesions, we detected the spontaneous formation of numerous large TRAP+ bone resorbing OCs with a longer survival whereas in the same type of culture derived from the MM patients without osteolyis and controls, we demonstrated smaller and fewer OCs not exhibiting a longer survival. On the other hand, in T cell-depleted MM PBMC cultures, exogenous macrophage-colony stimulating factor (M-CSF) and receptor activator of nuclear factor (NF)-κB (RANKL) were necessary to the formation of OCs, not displaying however a longer survival. Our cultures represent a good in vitro model to study the effect of MM T cells on osteoclastogenesis, being a MM and stromal cell-free system. RANKL appeared to be the major osteoclastogenic cytokine involved in the spontaneous OC formation, as confirmed by the inhibitory effect exerted on it by RANK-Fc. Besides RANKL, we detected the overexpression of osteoprotegerin (OPG) and TNF-related apoptosis inducing ligand (TRAIL) by fresh T cells isolated from the MM patients with lytic bone disease, at both mRNA and protein levels. All these cytokines were also detectable in the media collected from the unfractionated PBMC cultures. OPG is a soluble decoy receptor of TRAIL that competes with RANKL for binding to TRAIL, whose antagonizes the osteoclastogenic effect preserving the bone mass. In our culture system the persistence of osteoclastogenesis occurring in the presence of T cells, despite the high levels of OPG, could be explained by OPG binding to TRAIL, that may be favoured by the elevated TRAIL levels detected in the media. Moreover, neutralizing anti-TRAIL antibodies caused a dose-dependent inhibition of the spontaneous osteoclastogenesis, that recurred after the addition of exogenous RANKL. Thus, OPG/TRAIL interaction could inhibit both OPG anti-osteoclastogenic activity and TRAIL apoptosis-inducing activity. Our findings indicate a T cell-regulation of MM OC formation and survival through RANKL, OPG and TRAIL overexpression, possibly involving OPG/TRAIL interaction.

PO-090
IDENTIFICATION OF A NOVEL IGH-MMSET FUSION TRANSCRIPT IN A HUMAN MYELOMA CELL LINE WITH T(4;14) CHROMOSOMAL TRANSLOCATION


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Over the last few years, it has been shown that IGH translocation involving a wide range of partner loci are frequent events in plasma cell dyscrasias, being
present in approximately 50% of monoclonal gamopathies of uncertain significance, 60% of intramedullary multiple myeloma (MM), 70–80% of extramedullary form, and virtually all of human myeloma cell lines. The most frequent and well characterized IGH translocations include the t(11;14) (q13;q32), t(4;14)(p16.3;q32), t(14;16) (q32;q23) and t(6;14)(p21;q32) involving CCND1, FGFR3 and MMSET/WHSC1, MAF and CCND3 genes, respectively.

Three newly established human myeloma cell lines (KMS26, KMS28BM and KMS34) have recently been found to express high levels of FGFR3 transcripts by RT-PCR. The evidence of FGFR3 expression prompted us to investigate the presence of t(4;14) in these cell lines using dual-colour FISH and RT-PCR. The association of the signals specific for FGFR3 and MMSET and association of FGFR3 and the constant regions of IGH locus were detected in all three cell lines. The presence of IGH-MMSET fusion transcript was investigated by means of the RT-PCR using ms6r primer coupled with JH or Imu1 primers. The KMS34 and KMS26 cell lines showed IGH-MMSET fragments similar to those expected in the MB4-1 and MB4-3 type breakpoints. In the KMS28BM, an IGH-MMSET fragment smaller than that specific for the MB4-3 was detected with either JH-ms6r or Imu1-ms6r primers. The direct sequencing of this fragment revealed the presence of MMSET exon 6 sequences, but the absence of the entire MMSET exon 5, suggesting the occurrence of a novel breakpoint within intron 5 of the gene. The cloning of a genomic IGH rearranged fragments resulting in the translocation revealed that: 1) the switch mu region was joined to a switch γ1 sequence, thus suggesting that the recombination event between the IGH and 4p16.3 occurred after a legitimate class switch recombination; 2) the 4p16.3 sequence juxtaposed to switch γ1 was derived from a MMSET region located upstream of untranslocated exon 1 (205 nucleotides involving MMSET sequences containing translated exons 3–5). This novel fusion transcript represents a rare event since it was not detected in 8 HMCLs and 26 primary MMs with the t(4;14) investigated in our laboratory.

**Molecular and Biological Characterization of Three Novel Interleukin-6 Dependent Human Myeloma Cell Lines (CMA-01; CMA-02; CMA-03)**

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Human multiple myeloma cell lines (HMCLs) represent a very useful tool in the characterization of primary myeloma cells and in the understanding of some biological features of multiple myeloma (MM) such as interaction with growth factors, involvement in angiogenesis processes, response to anticancer drugs. Moreover, in the last years several chromosome translocations affecting the immunoglobulin loci (Ig) have been identified by molecular analysis in HMCLs. We report the establishment of three novel HMCLs (CMA-01; CMA-02; CMA-03) derived from malignant plasma cells at the extra-medullary phase of the disease. Peripheral mononuclear cells were cultured in IMDM supplemented with 10% FCS and 20U/ml recombinant human IL-6. After 30–40 days the cells started to grow and they are still maintained in the same culture conditions. The three cell lines were Epstein–Barr virus negative. Immunophenotypic analyses were performed by mean of FACS Calibur flow cytometry. In particular, all cell lines showed a bright expression of CD138 while CD126 is expressed at variable levels. CMA-01 cells expressed cytoplasmic and surface IgD lambda, CMA-02 expressed IgG kappa and CMA-03 expressed IgA kappa. Expression of CD45, CD56, CD20, CD19 and CD117 was also analysed. Cytogenetic analysis revealed a hypotetraploid complex karyotype with many numerical and structural abnormalities in all cell lines. FISH analysis was performed in order to investigate the presence of the most frequent translocations involving the IGH locus, found in MM. Using specific probes we detected t(11;14)(q13;q32.3) in CMA-01 and t(14;16) (q32.3;q23) in CMA-02 deregulating Cyclin D1 and MAF genes, respectively. The t(8;14)(q24;q32), involving the MYC locus, was present in all the three cell lines in complex chromosomal rearrangements. To characterize the proliferation pattern of the cell lines we analysed the growth curve and DNA distribution, the IL-6 dependency and the cloning efficiency in soft agar. The population doubling time was evaluated from the growth curve of cells exponentially proliferating and was estimated as 72, 110 and 84 hours, in CMA-01, CMA-02 and CMA-03 respectively. The cells grow at low saturation density (5 × 10^5 cells/mL) and are strictly dependent on IL-6 presence in culture medium. A low but appreciable cloning efficiency (about 1%) was detected in all three cell lines.
The mevalonate pathway yields to the synthesis of cholesterol, farnesyl pyrophosphate (FPP) and geranylgeranyl pyrophosphate (GGPP). FPP and GGPP play a key role in protein isoprenylation. This consists in the covalent transfer of farnesyl and geranyl geranyl groups from FPP and GGPP to regulatory proteins. After isoprenylation, proteins such as Ras, Rho and Rac translocate to the inner surface of plasma membrane and exert their regulatory activity on several cell functions. Zoledronic acid (Zol) is a potent inhibitor of FPP synthase. Zol belongs to the family of aminobisphosphonates (NBP) and is clinically used to treat bone lesions in multiple myeloma (MM) and other cancers. Inhibition of FPP synthase by Zol induces the intracellular accumulation of mevalonate metabolites such as isopentenyl-pyrophosphate (IPP). It has been shown that exogenous IPP activates the small subset of circulating gamma/delta T cells expressing the Vgamma9/Vdelta2 TCR. These are innate effector cells with the capacity to naturally recognize microbic components, structurally related to IPP. Upon activation, gamma/delta T cells exert cytotoxic activity against a variety of pathogens and certain tumor cells of hematopoietic origin. We have recently shown in normal donors and MM patients that Zol induces the proliferation of naive and memory gamma/delta T cells. When tumor cells were incubated with Zol in the presence of Mev, they became resistant to the effector functions of gamma/delta T cells. Thus, the immunomodulatory activity of Zol is also dependent on the mevalonate pathway of tumor cells. Interestingly, Zol-treated tumor cell lines showed different sensitivity to T cells, suggesting that this pathway can have different levels of activity in tumor cells and play a role in host immune recognition.

**PO-093**

**RANDOMIZED STUDY OF ZOLEDRONATE VS OBSERVATION IN PATIENTS WITH EARLY-STAGE, ASYMPTOMATIC MYELOMA: AN INTERIM REPORT**


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We have recently reported that prophylactic administration of pamidronate, a second generation bisphosphonate, is not useful to prevent or delay disease progression in patients with untreated, early-stage myeloma, although this treatment may reduce the number of cases developing skeletal events, thus possibly changing the clinical manifestations of the disease at progression (Musto et al., J Clin Oncol 2003; 21:3177–8, Musto et al., Leuk Lymphoma 2003; 44:1545–8). However, no data are currently available about the role of zoledronate, a more potent, third generation bisphosphonate, in this specific setting of patients. Therefore, on June, 2001, we started a randomized, multicentre trial to compare the effects of one-year administration of zoledronate as single therapy vs simple observation in asymptomatic
patients with monoclonal gammopathy fulfilling the diagnostic criteria of stage IA, IIA or smouldering myeloma, without evidence of bone lesions. Patients strictly diagnosed as having true MGUS were excluded. The aim of this study was to establish whether zoledronate may influence rate, time to and type of progression in these otherwise untreated patients. As of May, 2004, seventy-four patients (40 males, 34 females; mean age 67.4 years, range 41-85) have been enrolled and randomized (1:1) to receive (n. 37) or not (n. 37) zoledronate (Zometa, Novartis Pharmaceuticals, Origgio, Italy) for one year or until progression, on an out–patient basis, at the dose of 4 mg as 15’ i. v. single monthly infusion. Current median follow-up is 18 months. Until now, no severe adverse events have been recorded during the study. In the observational arm two patients were lost at follow-up after six and twenty months, respectively. Asymptomatic hypocalcemia, without need of interrupting the treatment and promptly corrected by substitutive therapy, occurred in nine of patients receiving zoledronate. To-date, no relevant reduction of M-component or marrow plasmocytosis has been observed. There have been four (10.8%) progressions requiring chemotherapy during follow-up in both arms, bone lesions and/or hypercalcemia at the time of progression were observed in 5/7 of controls, and in 1/4 of patients treated with zoledronate. Thus, in this interim analysis, a slight trend in favour of zoledronate–arm was seen. The next evaluations of our trial, im analysis, a slight trend in favour of zoledronate-treated patients. Thus, in this interim analysis, a slight trend in favour of zoledronate-arm was seen. The next evaluations of our trial, im analysis, a slight trend in favour of zoledronate-treated patients. Thus, in this interim analysis, a slight trend in favour of zoledronate-arm was seen. The next evaluations of our trial, im analysis, a slight trend in favour of zoledronate-treated patients. Thus, in this interim analysis, a slight trend in favour of zoledronate-arm was seen. The next evaluations of our trial, im analysis, a slight trend in favour of zoledronate-treated patients. Thus, in this interim analysis, a slight trend in favour of zoledronate-arm was seen. The next evaluations of our trial, im analysis, a slight trend in favour of zoledronate-treated patients. Thus, in this interim analysis, a slight trend in favour of zoledronate-arm was seen. The next evaluations of our trial, im analysis, a slight trend in favour of zoledronate-treated patients. Thus, in this interim analysis, a slight trend in favour of zoledronate-arm was seen. The next evaluations of our trial, im analysis, a slight trend in favour of zoledronate-treated patients. Thus, in this interim analysis, a slight trend in favour of zoledronate-arm was seen. The next evaluations of our trial, im analysis, a slight trend in favour of zoledronate-treated patients. Thus, in this interim analysis, a slight trend in favour of zoledronate-arm was seen.

PO-094
THE COMBINATION OF THIOTEPA, FLUDARABINE AND MELPHALAN IS EFFECTIVE AS REDUCED INTENSITY CONDITIONING FOR ALLOGENEIC TRANSPLANTATION IN MULTIPLE MYELOMA

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We have demonstrated that allogeneic transplantation is able to induce clinical and molecular remission in a high proportion of MM patients (Bone Marrow Transplant 2003;31:767-73). However, standard-dose conditioning still results in a high TRM. To reduce mortality, a low-intensity conditioning of fludarabine 3×30 mg/m², thiopeta 10 mg/kg and melphalan 80 mg/m² with HLA-identical sibling stem cell transplantation was designed. GVHD prophylaxis is low-dose MTX plus CSA, but the latter is rapidly tapered following transplantation. PCR analysis for IgH gene rearrangement is employed for MRD investigation. Up to now, 34 patients (38-68 y, median 53) were allografted, at 3-123 mo from diagnosis (median 11), 21 of them as late treatment after single (n=12) double (n=8) or triple (n=1) autograft and 12 as part of their front-line treatment, following a debulking with only 3-4 courses of VAD. At the time of conditioning, 8 were in CR, 17 in PR, 4 were refractory and 5 had progressive disease. They received 5.5×10⁶/Kg (median) CD34+ cells (range 1.6-10.6), and 2.8×10⁵/Kg CD3+ cells (range 0.4-6.1) from BM or G-CSF-primed PB. Engraftment occurred in all, with 14 days to >0.5×10⁹/L granulocytes (range 10-17) and 12 days to >20×10⁹/L platelets (range 4-21). aGVHD >grade I developed in 43% of evaluable pts, but it never was > grade II. cGVHD developed in 68%. Transplant response was assessed at day +90.19 out of the 27 (70%) evaluable pts were in CR, including the 9 who were already in CR at the time of allograft, while 5 acquired only a PR. Only 2 pts died for transplant-related causes (TRM=6%). Four patients relapsed and four are in progression after partial remission. 22 patients are currently alive at a median FU of 9.6 mo, 13 of them in continuous CR. OS is 57% and EFS is 30% at two years. Reduced-intensity conditioning with fludarabine, thiopeta and melphalan is well tolerated even in patients with previous autograft(s) and ensure a good remission of disease. Data of IgH–gene rearrangement are being produced and will possibly shed light on the significance of CR after this treatment.

PO-095
PROGNOSTIC ROLE OF MINIMAL RESIDUAL DISEASE IN MULTIPLE MYELOMA PATIENTS AFTER NON-MYELOABLATIVE ALLOGENEIC TRANSPLANTATION

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his study evaluates the prognostic value of molecular monitoring of minimal residual disease (MRD) in 20 patients with multiple myeloma (MM) treated by a tandem transplant program. The therapeutic strategy included autologous transplantation (PBSCT) followed by non-myeloablative allogeneic transplant (NMT). Half of patients were conditioned with fludarabine and cyclophosphamide and the remaining cases with fludarabine and low-dose TBI. All patients completed their program and engrafted; 9 patients (45%) developed aGVHD (grade 1-4) during the first 42 days of follow-up. Six patients (30%) developed cGVHD at a median of 145 days, with two cases that developed extensive cGVHD. After a median follow-up of 35 months, 7 patients (35%) had died, 3 of them because of disease progression. Actuarial incidence of GVHD was 20%, with no significant correlation with GVHD. The overall PFS at 24 months from the NMT was 520%, with no significant correlation with GVHD. The therapeutic strategy included autologous transplantation (PBSCT) followed by non-myeloablative allogeneic transplant (NMT). Half of patients were conditioned with fludarabine and cyclophosphamide and the remaining cases with fludarabine and low-dose TBI. All patients completed their program and engrafted; 9 patients (45%) developed aGVHD (grade 1-4) during the first 42 days of follow-up. Six patients (30%) developed cGVHD at a median of 145 days, with two cases that developed extensive cGVHD. After a median follow-up of 35 months, 7 patients (35%) had died, 3 of them because of disease progression. Actuarial incidence of GVHD was 20%, with no significant correlation with GVHD. The overall PFS at 24 months from the NMT was 51%. In univariate analysis, a shorter PFS was associated with advanced stage at diagnosis (p=0.03) and with failure of PBSCT (p=0.048). The allogeneic procedure resulted offer a higher MRD eradication rate than autologous transplantation: after PBSCT, only 3 patients (15%) achieved PCR-negativity, versus 11 (61%) after NMT. The IgH rearrangement, evaluated by fluorescent PCR, was adopted as surrogate for MRD status. MRD after allogeneic graft did not significantly correlate either with clinical response or with chimerism. Seventy-five percent of patients achieved full donor chimerism, which was more frequently observed in cases presenting cGHVD (p=0.01) and positively associated with overall survival (OS). The eradication of MRD had a favorable impact on 2-year OS. In fact, 76% of patients with no detectable MRD were still alive versus 34% of persistently IgH-positive cases (p=0.03). Finally, MRD did significantly correlate with disease progression, considering that all progressed cases resulted PCR-positive versus 43% of patients with stable disease (p=0.02). These data sustain the relevant role of molecular monitoring in MM patients undergoing NMT. MRD monitoring would assist physicians in making additional therapeutic decisions to better control this haematological malignancy.

**PO-096**

**Phase I-II Trial of Anti-Cancer Vaccination for Multiple Myeloma Patients Using Dendritic Cells Pulsed with Tumor Idiotype (ID) Or Id (VDJ)-Derived Peptides**


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Thirteen multiple myeloma (MM) patients were treated with two courses of high-dose chemotherapy with peripheral blood stem cell support and then entered in a clinical study of anti- Id. vaccination with dendritic cells (DC). DC were generated from positively selected circulating monocytes according to good manufacturing practice guidelines, in FCS-free medium in cell culture bags, in presence of GM-CSF plus IL-4 followed by either TNF-α or a cocktail of IL-1-β, IL-6, TNF-α and prostaglandin-E2. CD14+ monocytes were enriched from 16.1±5.7% to 95.5±3.2% (recovery 67.9±15%, viability >97%). After cell culture, phenotypic analysis showed that 89.67±6.6% of the cells were DC; we obtained 2.89±3.1×10⁸ DC/leukapheresis which represented 24.5±9% of the initial number of CD14+ cells. Notably, the cytokine cocktail induced a significantly higher percentage and yield (31±10.9 of initial CD14+ cells) of DC than TNF-α alone, secretion of larger amounts of IL-12, potent stimulatory activity on allogeic and autologous T cells. Storage in liquid nitrogen did not modify the phenotype or functional characteristics of pre-loaded DC. The recovery of thawed, viable DC, was 78±9.6%. Ten patients in partial remission after autologous stem cell transplantation received a series of by-monthly immunizations consisting of three subcutaneous and two intravenous injections of Id-keyhole limpet hemocyanin (KLH)-pulsed DC (5×, 50×10⁶ cells and 10×, 50×10⁷ cells, respectively). The patient-specific Id was used as whole protein in 4 patients whereas 6 additional patients had their DC charged with Id (VDJ)-derived HLA class I restricted peptides. The administration of Id-pulsed DC was well tolerated with no clinically significant side effects. So far, 6 patients have been fully evaluated for their immunologic response to DC vaccination. Six of 6 patients developed a humoral and T-cell proliferative response to KLH. Moreover, 5/6 showed circulating IFN-γ-secreting T cells by Elispot. None of the patients mounted a B-cell response to Id whereas 6/6 developed a Id-specific T-cell proliferative response and 4/6 IFN-γ-secreting T cells. Delayed-type hypersensi-
tivity (DTH) tests showed 6/8 and 2/8 patients responsive to KLH and tumor Id, respectively. Ten out of thirteen patients have completed vaccination schedule. With a median follow up of 20 months, 4/10 patients have stable disease, 2 patients are in CR, 1 patient obtained clinical response and 3 patients progressed. In summary, positive selection of circulating CD14+ monocytes allows the generation of mature and functional DC suitable for clinical trials and cryopreservation does not affect the phenotype and function of pre-loaded DC. Moreover, injections of cryopreserved DC pulsed with tumor Id or Id-derived peptides are safe and induce T-cell tumor-specific responses.

In multiple myeloma patients, adoptive immunotherapy through allogeneic donor lymphocyte infusions resulted in well-documented graft versus myeloma effect, but it is associated with high incidence of graft versus host disease. The idiotype (Id) expressed by MM cells can be regarded as a tumor-specific antigen and it has been used for immunotherapy. To enhance antitumor immunity and reduce alloreactivity, allogeneic T cells were activated against tumor-derived Id and then purified according to IFN-gamma production. Total peripheral blood mononuclear cells from healthy donors were incubated with autologous monocyte-derived dendritic cells generated in the presence of GM-CSF and IL-4 and pulsed with patient-derived Id protein. Cells were maintained in serum-free medium and supplemented during the priming phase with IL-7 and IL-12. Subsequently, the T-cell culture was restimulated every 7 days with pulsed DC in the presence of low doses of IL-2. After 2 or 3 stimulations, the percentage of IFN-gamma-producing T cells was as high as 5-10%, whereas that observed in presence of not stimulated T-cells was undetectable. Based on their IFN-gamma production, T cells were isolated by using a commercial immunomagnetic IFN-gamma capture assay. The purity of enriched IFN-gamma-producing T cells ranged between 60 and 90% as evaluated by flow cytometry. The yield was 60% of pre-selection IFN-gamma positive T cells and cell viability after selection was 80%. Functionally, IFN-gamma purified T cells showed better Id-specific proliferative response when compared both to not-stimulated T cells and to stimulated but not purified T cells. Moreover, addition of not-stimulated T cells resulted in significant reduction of allogeneic CD34+ derived colony-forming capacity. Conversely, the absolute number of allogeneic total colony-forming units–cells was not affected by IFN-gamma purified T cells. These data demonstrate that Id-specific T cells may be generated from healthy donors and significantly enriched on the basis of their IFN-gamma production. Id-specific IFN-gamma purified T cells have better anti-Id response and reduced alloreactivity than unselected T cells.

PO-097
PURIFICATION OF ALLOGENEIC IDIOTYPE-SPECIFIC T LYMPHOCYTES ACCORDING TO IFN-γ PRODUCTION FOR ADOPTIVE IMMUNOTHERAPY IN MULTIPLE MYELOMA PATIENTS
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In multiple myeloma patients, adoptive immunotherapy through allogeneic donor lymphocyte infusions resulted in well-documented graft versus myeloma effect, but it is associated with high incidence of graft versus host disease. The idiotype (Id) expressed by MM cells can be regarded as a tumor-specific antigen and it has been used for immunotherapy. To enhance antitumor immunity and reduce alloreactivity, allogeneic T cells were activated against tumor-derived Id and then purified according to IFN-gamma production. Total peripheral blood mononuclear cells from healthy donors were incubated with autologous monocyte-derived dendritic cells generated in the presence of GM-CSF and IL-4 and pulsed with patient-derived Id protein. Cells were maintained in serum-free medium and supplemented during the priming phase with IL-7 and IL-12. Subsequently, the T-cell culture was restimulated every 7 days with pulsed DC in the presence of low doses of IL-2. After 2 or 3 stimulations, the percentage of IFN-gamma-producing T cells was as high as 5-10%, whereas that observed in presence of not stimulated T-cells was undetectable. Based on their IFN-gamma production, T cells were isolated by using a commercial immunomagnetic IFN-gamma capture assay. The purity of enriched IFN-gamma-producing T cells ranged between 60 and 90% as evaluated by flow cytometry. The yield was 60% of pre-selection IFN-gamma positive T cells and cell viability after selection was 80%. Functionally, IFN-gamma purified T cells showed better Id-specific proliferative response when compared both to not-stimulated T cells and to stimulated but not purified T cells. Moreover, addition of not-stimulated T cells resulted in significant reduction of allogeneic CD34+ derived colony-forming capacity. Conversely, the absolute number of allogeneic total colony-forming units–cells was not affected by IFN-γ purified T cells. These data demonstrate that Id-specific T cells may be generated from healthy donors and significantly enriched on the basis of their IFN-γ production. Id-specific IFN-gamma purified T cells have better anti-Id response and reduced alloreactivity than unselected T cells.
PO-098
DUAL EFFECT OF ARSENIC TRIOXIDE ON HEMOPOIESIS: INHIBITION OF ERYTHROPOIESIS AND STIMULATION OF MEGAKARYOCYTOPOIESIS
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Arsenic compounds, including arsenic trioxide (As2O3) and arsenic disulfide, utilized in some traditional Chinese remedies, have been demonstrated to be effective for the treatment of Acute Promyelocytic Leukemia (APL), when used at low doses. However, As2O3 is also a potent inducer of apoptosis in a number of other cancer cells such as AML, gastric cancer, neuroblastoma. The exact mechanism of As2O3 induced apoptosis in the cells is not yet clear. In the present study we investigated the effect of As2O3 on erythropoiesis and megakaryocytopoiesis. During As2O3 treatment of the human erythroleukemic cell line HEL several changes were observed: stimulation of megakaryocytic differentiation, inhibition of several erythroid markers and induction of apoptosis at the same time. Particularly, the expression of erythroid-specific receptors such as c-Kit, Epo-R, GlycA, were downmodulated after As2O3 exposure. Similar observations have been made in K562 cells. The same phenomenon was observed during unilineage erythroid and megakaryocytic cultures from normal hemopoietic progenitor cells: blockade of erythropoiesis and megakaryocytopoiesis. During As2O3 treatment of the human erythroleukemic cell line HEL several changes were observed: stimulation of megakaryocytic differentiation, inhibition of several erythroid markers and induction of apoptosis at the same time. Particularly, the expression of erythroid-specific receptors such as c-Kit, Epo-R, GlycA, were downmodulated after As2O3 exposure. Similar observations have been made in K562 cells. The same phenomenon was observed during unilineage erythroid and megakaryocytic cultures from normal hemopoietic progenitor cells: blockade of erythropoiesis and megakaryocytopoiesis. As shown by cell cycle, morphologic and immunophenotypic analyses. To determine whether the engagement of caspases is involved in these phenomena we analyzed the expression of GATA-1 and Tal-1 transcription factors, whose activity is essential for the development and survival of the erythroid lineage. After exposure of HEL and K562 cells to As2O3 for 48h we observed a decrease of GATA-1 and Tal-1 expression; moreover, treatment with pan-caspase inhibitor z-VAD in combination with As2O3, protects GATA-1 from cleavage. In unilineage cultures of normal erythroid progenitors the addition of As2O3 resulted in a blockade of erythroid maturation at the proerythroblast stage, associated with a cleavage of both GATA-1 and Tal-1 transcription factors. Both the As2O3-induced inhibition of erythroid cell differentiation and cleavage of transcription factors was blocked by zVAD. In contrast, the addition of As2O3 to unilineage cultures of normal megakaryocytic precursors did not induce any cleavage of GATA-1 and Tal-1 transcription factors and induced a stimulation of megakaryocytic maturation (i.e., increase in the formation of the large polyploid megakaryocytes). The stimulatory effect of As2O3 on megakaryocytic maturation seems to require caspase activation. Experiments are in progress to determine the caspase required for the inhibitory and stimulatory effect of As2O3 on erythropoiesis and megakaryocytopoiesis, respectively. Our results clearly indicate that the effects of As2O3 on hematoipoiesis are lineage specific and the marked inhibitory effect of this compound on erythropoiesis could explain the development of anemia often observed during therapy based on As2O3 administration.

PO-099
USE OF THE BLOB ANALYSIS IN THE DEVELOPMENT OF A NEW PROCEDURE FOR THE AUTOMATIC COUNT OF GRANULOCYTES MIGRATING THROUGH MICROPOROUS FILTERS IN CHEMOTACTIC BOYDEN CHAMBER
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In this paper we describe a completely new image processing procedure for an automatic assessment of granulocyte motility in micropore filters. Granulocyte motility was evaluated in perspex chemotactic chambers according to the Boyden method, using mixed-ester filters between the two compartments. After treatment, the filters were fixed, dehydrated, stained, diaphanized and placed on the electromechanical table of a Leitz Hortolux microscope. According to our procedure, the objective is focused on the upper plane of the filter and a sequence of digital images is then acquired at predetermined depths by means of a video camera. The video signal is sent to a video board which can store images defined by 768×576 pixels with 300 μm spatial resolution. The frame grabber works on a personal computer under the control of the Matrox Inspector(R) software, which uses several options, such as Seg-
mentation, Setting and Features. Initially, a decision threshold is properly selected, according to a greyscale segmentation threshold. Then, regions of touching pixels are automatically identified by the software as a blob, each blob corresponding to a cell in the processed image. Setting allows the definition of the Foreground and the option Remove Blobs regards the situation of Touching Blobs, or the definition of Minimum Area or Maximum Area (expressed in pixel²). Each image is processed in order to reveal the blobs, produced by the granulocytes, and to extract the plane coordinates (x,y) of the centroid of each blob. The results obtained by processing the first image of the sequence are compared with the results obtained by processing the second one, in order to detect couples of blobs having the same plane coordinates and to delete one of them. This comparison is performed considering the results obtained from second and third images, and so on. The complete procedure is performed in few seconds. The automatic analysis was compared blind with a traditional analysis performed by an optical detection of the cells in each plane: no statistical differences were found between the two sequences, except for the plane corresponding to 20 µm, which resulted critical in the visual way, showing a bias due to the focusing of a large amount of cells under examination, which leads to count twice the same cells. On the contrary the software was able to eliminate this artifactual effect, by recognizing each cell and assigning it to the plane in which it really is. So the procedures performs a reliable and reproducible count of the of the granulocytes in the micropore filters (without underestimation or overestimation) and determines not only the depth reached by the cells, but also their true propagation curve.

PO-100
IMMUNOREGULATORY EFFECT OF PR-39, AN ANTIMICROBIAL PEPTIDE
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Introduction. Neutrophil antimicrobial peptides are contained in azurophilic grains of the neutrophil and have microbicidal properties. They are also secreted by many epithelial cells, and many antimicrobial peptides have additional effects on innate immunity. PR-39 is a proline-arginine rich antimicrobial peptide, belonging to the sub-family of the cathelicidins. PR-39 was cloned in 1993 from the pig neutrophil. PR-39 blunts neutrophil activation by binding to intracellular SH3 domains during NAD(P)H oxidase assembly. The evidence for the existence of a neutrophil-type NAD(P)H oxidase in platelets, has been confirmed by the identification of the subunit p67phox, p47phox and a gp91phox. Platelets (PLTs) produce reactive oxygen subspecies (ROS) that stimulated platelet aggregation and the interaction with neutrophils. PLTs activation is thought to be a key in acute vascular thrombosis. Therefore, prevention of enhanced platelet activation is a major target of therapeutic strategies fighting cardiovascular and cerebrovascular diseases. Since the platelet NAD(P)H oxidase presents a potential target for PR-39, the aim of our study was to verify if the PR-39 interferes with activation, aggregation, production of platelet ROS and platelets-neutrophil interaction. Methods. We studied the effect of 2 concentrations of PR-39 (5µM, 1µM) on platelet function. Platelet Rich Plasma (PRP) or whole blood, incubated with or without PR-39, was tested for aggregation, ROS production and PLT-PMN interaction with and without stimulus. Dihydrododamine-123 (DHR-123) was used to examine in vitro platelets ROS production with a flow cytometric method. 12-phobol-13-myristate acetate (PMA), and f-met-leu-phe (fMLP) were used as agonists. Data are expressed as fluorescence mean intensity (FMI). PLT-PMN complexes were measured using flow cytometry. Data are expressed as percent of neutrophils which co-localized with PLT markers under basal condition and after blood stimulation with ADP, fMLP or PMA. Platelet aggregation was studied used PRP stimulated with ADP or thrombin in a CHRONOLOG aggregometer. Results. PR-39 had no affect on platelet aggregation. PR-39 at 1 µM had no affect on platelet ROS production while PR-39 5 µM inhibited fMLP- (5.307 ±0.729 vs 3.589 ±0.885, p<0.05) and PMA-stimulated ROS production (1471 ±1.356 vs 8.20 ±1.4, p<0.01). PMN-PLT interaction under basal condition increases in presence of ADP (31.16%±9.54 vs 49.08%±16.09) and PMA (31.16%±9.54 vs 69.73±6.75, p<0.05). PR-39 pre-incubation inhibits this effect at 1 and 5 µmolar concentration (p<0.05). Conclusion. PR-39 blunts PLT ROS production and PMN-PLT aggregation. Although PLTs don’t produce large amounts of reactive oxygen compared to PMNs, even small amounts of ROS may initiate pro-inflammatory signalling processes. Therefore, detailed description of relevant sources of ROS in postischemic tissue, and the elucidation of regulatory mechanisms, is critical to our understanding of the steps leading to pro-coagulatory effect and on inflammatory injury.
PO-101
A WHOLE BLOOD FLOW CYTOMETRIC METHOD TO EVALUATE F-ACTIN POLYMERIZATION IN NEUTROPHILS

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To reduce artifactual effects in the study of F-actin dynamics in neutrophils, we have developed a whole blood flow cytometric method. Both isolated and whole blood neutrophils were studied under unstimulated conditions (at 4°C and 37°C) and after stimulation with 10 nM f-MLP for 15, 30, 60 and 120 sec. Cells were permeabilized by formalin and lysophosphatidyl choline and stained with FITC-phallacidin. The whole blood method did not modify significantly the scatter properties of whole blood leukocytes and allowed a rapid analysis of F-actin polymerisation. F-actin content was measured after normalization of flow cytometry results, to obtained semiquantitative values and avoid differences caused by the use of different cytometers and operating approaches. F-actin polymerisation dynamics was very similar when evaluated by the two different methods, without any significant differences in F-actin relative content. In addition, we found that differences between F-actin content at 37°C and F-actin content at 4°C represent the spontaneous capability of polymerising actin: in fact, alternating exposure of neutrophils at 4°C and 37°C (up to four consecutive cycles) induced alternating phenomena of polymerisation and depolymerisation. Thus, our whole blood method seems to be able to evaluate not only F-actin polymerisation after chemotactic stimulus with f-MLP, but also the intrinsic capability of polymerising actin under ex-vivo conditions. This method can be very useful to evaluate this important phenomenon when the effects of drugs and growth factors have to be evaluated.

PO-102
ANTINFAMMATORY PROPERTIES OF ANTITHROMBIN-III

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Background. Current interest focused on the interrelation between inflammation and coagulation, and we have studied the anti-inflammatory properties of antithrombin-III (AT-III). Cardiac surgery involving cardiopulmonary bypass leads to fulminant activation of the hemostatic-inflammatory system. Transgenic recombinant human antithrombin III (rhAT-III) is in phase III clinical trials in the US and Europe as an anti-coagulant and anti-inflammatory agent in patients undergoing elective cardiac surgery with cardiopulmonary bypass. Effects of AT-III supplementation on the inflammatory response during cardiac surgery are less well investigated. Cardiopulmonary bypass is associated with extensive granulocyte and monocyte activation including the release of pro- and anti-inflammatory cytokines and up-regulation of adhesion molecules including CD11b/CD18 which is responsible for firm leukocyte adhesion to platelets and fibrinogen. Blood contact with artificial surfaces decreases the ability of activated platelets, leukocytes and their aggregates to pass through organ capillaries, and neutrophils sequestration has been implicated in the inflammatory response associated with cardiopulmonary bypass. AT-III attenuates ischemia-induced leukocyte adhesion and might be able to directly affect properties of leukocytes during extracorporeal circulation. Methods. We studied CD11b/CD18 expression on PMN in flow cytometry. We performed the in-vitro analysis on basal (B group) and activated after incubation (20 minutes, 37°C) with formyl-Met-Leu-Phe (fMLP, 10e-6 M) and/or hrAT-III (1 U/ml) expression. We pre-incubated whole blood with hrAT-III (20 minutes, 37°C), and then we stimulated PMN with fMLP (20 minutes, 37°C). Leukocyte aggregation was monitored as the increase in transmission of light through stirred suspension in a platelet aggregometer in the first 6 minutes following stimulation. Leukocytes in platelet-rich-plasma (LPRP) were obtained from heparinized whole blood by centrifugation. Aggregation was induced by phytoemagglutinin (PHA, 0.24 mg/mL) + hrAT-III at various concentration, or with PHA alone after cells incubation (20 minutes, 37°C) with hrAT-III at various concentration. NADPH oxidase activity in PMN, was studied using a fluorescent dye suitable for flow cytometry, Dihydrorhodamine 1,2,3 (DHR123). Blood samples were preincubated (20 minute, 37°C) with hrAT-III (2.5 IU/mL). We analysed NADPH oxidase activity using PMA 100 ng/mL and fMLP 1 microM as stimuli. Results. After stimulation with fMLP, we confirmed a significant increase in CD11b/CD18 expression on PMN versus the basal. We observed that AT-III was unable to affect in vitro expression of integrins, while AT-III pre-incubation inhibited the fMLP-induced CD11b/CD18 up-regulation. At the basal condition, fMLP and PMA induced a significant enhance in free radical production, whereas hrAT-III alone did not activate NADPH oxidase activity; hrAT-III pre-incubation inhibited the PMA-induced NADPH oxidase activation but did not have any effect on fMLP. In the presence of plasma and platelets, aggregation of nor-
Women with Von Willebrand Disease (VWD) type 1 and 2 usually show increasing von Willebrand factor (VWF) levels during pregnancy, due to the constitutive release of VWF by the highly vascularized foeto-placental unit. Patients with type 2M Vicenza are characterized by very low plasma levels of VWF activities in the presence of supranormal multimers similar to those found in platelet α granules and endothelial Weibel-Palade bodies. Since the original description by Rodeghiero, many other families have been reported in different countries (Germany, USA, UK) and two distinct mutations have been identified (R1205H, M740I). However the basic mechanisms of this VWD variant remain not completely understood despite many attempts to study these recombinant VWF mutants after their expression in vitro. We had recently the opportunity to follow the pregnancy of a 37 year-old woman with Type 2M Vicenza R1205H.

Before pregnancy, she was exposed to an infusion trial with desmopressin (DDAVP). During pregnancy, she has been followed with monthly measurements of FVIII/VWF activities and multimeric analysis of VWF. Since VWF plasma levels were < 6 U/dL, the VWF:RCo was tested by our sensitive ELISA method. Basal levels of BT and FVIII/II/VWF activities were the same during the last five years (mean values): BT = 6.30 minutes; FVIII:C=18 U/dL VWF:Ag=5 U/dL; VWF:RCo = 3 U/dL, with normal VWF platelet content and supranormal multimers in plasma. She was considered responsive to DDAVP because FVIII:C, VWF:Ag and VWF:RCo were still relatively high at 2 hours post-DDAVP with values of 62, 36 and 32 U/dL respectively. Conversely, the levels of the FVIII/VWF activities measured at month 3, 6, 9 did not change at all during pregnancy. These clinical data might support the hypothesis raised by several authors that this type 2M VWD variant is due to defects of constitutive release of VWF.

PO-04
CHRONIC IMMUNE THROMBOCYTOPENIC PURPURA (ITP):
THE ROLE OF PLATELET APOPTOSIS AND DENDRITIC CELLS

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Chronic idiopathic thrombocytopenic purpura (ITP) is an autoimmune disorder characterized by platelet destruction via antiplatelet autoantibody. New mechanisms of autoimmunity have been recently described. A growing body of evidence supports the role of dendritic cells (DCs) in the pathogenesis of autoimmunity. Remarkably, recent results also suggest that, in particular circumstances, cells dying by apoptosis may trigger a specific immune response. In the present study, we were aimed to investigate whether platelet apoptosis and/or DCs may have a role in the stimulation of the immuno-mediated anti-platelet response in course of ITP. We studied 10 patients with active ITP and 8 healthy subjects. We found that ITP platelets, either fresh or in vitro aged for 3 days at 37°C in a plasma-free buffer, show a higher expression of phosphatidylinerine in comparison with their normal counterpart. We therefore investigated the role of caspases in the onset of platelet apoptosis and we found that caspases are not involved. We found that the expression of the pro-apoptotic proteins BAX, BAD and BAK in normal and ITP platelets did not significantly change during storage. Furthermore, we failed to detect any significant difference between normal and ITP platelets (either fresh or aged). We were also aimed to investigate whether phagocytosis of autologous fresh and in vitro aged platelets, either from healthy subjects or from ITP patients, leads to the processing/presentation of platelet antigens by DCs and the cross-presentation of T lymphocytes. In order to evaluate if platelets with apoptotic-associated signals (i.e., phosphatidylinerine) are able to induce their uptake by DCs, we firstly double-stained ITP aged platelets with PKH-26 (red fluorescence)/CD41a-FITC and we co-cultured them with immature DCs. Monocyte-derived DCs readily ingest aged platelets, as documented by flowcytometry analysis. We studied concentration, phenotype and function of monocyte-derived DCs. We found that the cells expressed all surface markers of mature DCs (CD1, CD83*, CD40+, CD86*, CD80*, HLA-DR*, CD14+) even though ITP DCs showed a significantly higher expression of the co-stimulatory molecule CD86. Remarkably, we were able to show...
that ITP DCs, after pulsing with autologous fresh or aged platelets, stimulated more efficiently autologous and allogeneic T cell proliferation than their normal counterpart. This has to be referred, at least in part, to the significantly higher expression of CD86 co-stimulatory molecule in ITP DCs. When we studied the effect of allogeneic fresh and aged platelets on the stimulation of lymphocyte proliferation by pre-pulsed DCs, once again we found that ITP DCs are more efficient to present aged platelet antigens than the normal ones. This finding suggests that the T lymphocyte proliferation is due to the increased antigen presentation capacity of ITP DCs and, to a lesser extent, to the apoptosis of ITP platelets. Furthermore, due to CD86 up-regulation in ITP DCs, our results suggest also that CTLA-4 targeting may be a new approach for future therapeutic purposes of ITP.

PO-105
ELEVATED PLASMA PROCOAGULANT AND ACTIVATION PEPTIDES MARKERS IN PATIENTS WITH LUNG CANCER

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Activation of coagulation and fibrinolysis within tumour tissues is thought to be associated with tumour growth, angiogenesis, and metastasis; alterations in haemostatic system are seen frequently in lung cancer correlated with the prognosis of disease. The aim of our study was to detect the hypercoagulability in patients with lung cancer as well as to search for a specific marker indicating the risk of DVT and PE related to tumour and chemotherapy.

We studied 33 patients with histologically confirmed lung cancer, there were 29 men (88 per cent) and 4 women (12 per cent) with a mean age of 60 years, 5 patients received chemotherapy. The plasma levels of the following markers were assessed: prothrombin time (PT), active partial thromboplastin time (aPTT), Factor VIII, von Willebrand Factor (vWF), tissue Plasminogen Activator (t-PA), Plasminogen Activator Inhibitor (PAI-1), prothrombin activation fragment (F1 + 2), D-dimer. MTHFR (C677T), Factor II (G20210A) and Factor V (G1691A) polymorphisms were also tested in 12 patients. The members of the study were divided into two groups as patients receiving or not chemotherapy. There was a statistically significant increase in Factor VIII, von Willebrand Factor (vWF), prothrombin activation fragment (F1+2) and D-dimer plasma levels in patients compared with controls (170±45; 172±40; 3.2±0.8; 3.6±0.9 vs 100±20; 105±21; 0.48±0.02; 1.45±0.2). Chemotherapy administration was associated with a higher increase of these markers (192±50; 181.2±45; 3.6±0.8; 6.0±2.0). Tissue Plasminogen Activator (t-PA), Plasminogen Activator Inhibitor (PAI-1) levels were decreased into the two groups compared with controls (2.0±0.3; 55±5.0 vs 7.5±0.5 32.5±1.5). In seven patients Methylentetrahydrofolate reductase (MTHFR) polymorphism was found. The results of our study shown here are indicative of an acquired thrombophilia in patients with lung cancer, enhanced by chemotherapy administration. The molecular basis of this thrombophilic state could be in part due to a disturbance in folate metabolism associated with an overexpression of coagulation factors genetically controlled by cancer cells. Our findings also demonstrate an endothelial dysfunction due to thrombin formation and fibrin deposition into vessel wall by neoplastic cells.

PO-106
HELICOBACTER PYLORI INFECTION IN CHRONIC IMMUNE THROMBOCYTOPENIC PURPURA

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Helicobacter pylori (HP) has been suspected to be involved in various autoimmune disorders including pernicious anemia and immune thrombocytopenic purpura (ITP). Several uncontrolled studies supported a pathogenic role of HP in ITP since they showed a 30-70% prevalence of HP infection in ITP and a partial or complete platelet recovery after bacterium eradication. On the contrary three additional studies, one of which was prospective, failed to confirm this association. We performed a prospective study in order to investigate the prevalence of HP infection in patients with ITP and the effect of its eradication on platelet count. Since September 2003, thirty-two consecutive adult patients admitted to our ward for ITP were enrolled in the study. Twenty-three patients had chronic ITP while nine had the acute form (disease duration lower than 6 months); 23 were females and 9 were males with a mean age of 51 years±19 (SD) and with a mean platelet count of 56±42x10^9/L. HP infection was found in 19 patients (59%) by means of the stool antigen test. HP-positive and negative patients were comparable for age, gender, platelet count, disease duration and therapy regimens. Six HP-positive patients were eligible to the eradication since their platelet count was more than 20x10^9/L and they do not need for starting or modifying therapy of thrombocytopenia for at least 3 months. They received triple therapy with omeprazole
20 mg twice daily plus clarithromycin 500 mg twice daily and amoxicillin 1 gr twice daily for 7 days, and the bacteria was eradicated in all. After 6 months of follow-up, a platelet count increase more than 50% of the initial count was observed in three patients (50%). In conclusion, we observed a higher-than-expected prevalence of HP in patients with ITP.

PO-107
PREVALENCE AND NATURAL HISTORY OF HEPATITIS VIRUS C INFECTION IN 350 ITALIAN PATIENTS WITH VON WILLEBRAND DISEASE

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Hepatitis C is a major cause of morbidity and mortality in patients with bleeding disorders who received clotting factors concentrates before the availability of virus-inactivated factors in the mid 1980s. Compared to hemophiliacs, patients with von Willebrand disease (VWD) have been less extensively exposed to large-pool concentrates because they could be treated by desmopressin or cryoprecipitates prepared by national blood banks. To assess the prevalence and natural history of HCV infection in VWD, 350 patients attending the ABB Hemophilia Thrombosis Center in Milan were enrolled in a cohort (types 1=145, 2=184 and 3=21). The 133/350 patients (37% males, with types 1=145, 2=184 and 3=21) who had been firstly exposed to cryoprecipitate and a full set of multimers. GpIb binding assay (T

Tabelle 1: Values of circulating vWF concentrations in patients with VWD types 1, 2B and 3

<table>
<thead>
<tr>
<th>VWF</th>
<th>WT</th>
<th>P1337L</th>
<th>P1337L/WT</th>
<th>C275R</th>
<th>P1337L/C275R</th>
</tr>
</thead>
<tbody>
<tr>
<td>Ristocetin 0,125 mg/mL</td>
<td>0.084</td>
<td>0.097</td>
<td>0.070</td>
<td>1.105</td>
<td>0.924</td>
</tr>
<tr>
<td>Ristocetin 0,25 mg/mL</td>
<td>0.107</td>
<td>0.154</td>
<td>0.140</td>
<td>0.924</td>
<td>0.924</td>
</tr>
<tr>
<td>Ristocetin 0,5 mg/mL</td>
<td>0.154</td>
<td>0.160</td>
<td>0.154</td>
<td>0.924</td>
<td>0.924</td>
</tr>
<tr>
<td>Ristocetin 1 mg/mL</td>
<td>0.215</td>
<td>0.220</td>
<td>0.215</td>
<td>0.924</td>
<td>0.924</td>
</tr>
</tbody>
</table>

It seems that rVWF-C275R molecules do not contribute with the rVWF-P1337L to form multimers.
Interleukin-10 (IL-10) is a key cytokine that is elevated during systemic inflammation. The data currently suggest that IL-10 is synthesized by various leukocytes and is important in regulating the production of other cytokines (interferon-γ, TNF-β and IL-2) by TH1 cells. IL-10 has been found to inhibit the antigen presenting cell-mediated stimulation of TH1 cells by inhibiting the production of inflammatory cytokines (IL-1α, IL-6, IL-8 and GM-CSF). Calcineurin inhibitors, namely Tacrolimus (FK) and Cyclosporine (CsA) share similar physicochemical properties and a common mechanism of action. FK in addition shows a strong anti-inflammatory property blocking the secretion of pro-inflammatory cytokines and an indirect inhibitory effect on the growth and differentiation of B lymphocytes. With this background we carried out serological studies in a consecutive refractory ITP patients receiving low-doses FK. Five patients were enrolled in the study. Median age was 50 yrs (range 23–63), M/F=3/2, median time from diagnosis was 4 months (range 1–24). The drug was given orally twice a day in order to maintain blood levels between 5–15ng/ml. 5/5 patients (100%) achieved CR (>1109/L for >12 weeks). The median time to CR was 5 months (range 1–9). In order to evaluate the response duration, all patients stopped FK therapy after a median of 5 months from CR (range 2–12). 2 pts relapsed after 2 and 1 patient after 3 months. All of these patients resumed FK therapy and re-achieved CR after 2 months. 1 patient lost CR but remained in PR (>40×9/L for >8 weeks) at 10 months follow-up; 1 patient showed a sustained CR (more than 20 months follow up). Cytokine levels were quantified by enzyme-linked immunosorbent assay (ELISA) technique with kits obtained from R&D Systems (Minneapolis, MN, USA). Serum γ IFN, IL-2 and s-IL-2R levels were also quantified. All samples showed IL-10 serum high levels (>500 pg/mL, range 7.8–500 pg/mL). Interesting, serum γ IFN and IL-2 were below defined cut-off (15 pg/ml and 1.2 U/ml) in all pts and serum levels IL-2 receptor were increased (cut-off 70 pg/ml). Our data suggest that IL-10 may be involved in limiting and terminating inflammatory responses in ITP patients. In this way IL-10 may be synergistic with FK anti-inflammatory property. FK, on the other hand, inhibits regulatory IL-10 effects on growth and/or differentiation of B cells.

**PO-109**

**INTERLEUKIN-10 (IL-10) SERUM LEVELS MAY BE SYNERGISTICS WITH LOW-DOSE TACROLIMUS (FK) IN THE TREATMENT OF REFRATORY IDIOPATHIC THROMBOCYTOPENIC PURPURA**

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Interleukin-10 (IL-10) is a key cytokine that is elevated during systemic inflammation. The data currently suggest that IL-10 is synthesized by various leukocytes and is important in regulating the production of other cytokines (interferon-γ, TNF-β and IL-2) by TH1 cells. IL-10 has been found to inhibit the antigen presenting cell-mediated stimulation of TH1 cells by inhibiting the production of inflammatory cytokines (IL-1α, IL-6, IL-8 and GM-CSF). Calcineurin inhibitors, namely Tacrolimus (FK) and Cyclosporine (CsA) share similar physicochemical properties and a common mechanism of action. FK in addition shows a strong anti-inflammatory property blocking the secretion of pro-inflammatory cytokines and an indirect inhibitory effect on the growth and differentiation of B lymphocytes. With this background we carried out serological studies in a consecutive refractory ITP patients receiving low-doses FK. Five patients were enrolled in the study. Median age was 50 yrs (range 23–63), M/F=3/2, median time from diagnosis was 4 months (range 1–24). The drug was given orally twice a day in order to maintain blood levels between 5–15ng/ml. 5/5 patients (100%) achieved CR (>1109/L for >12 weeks). The median time to CR was 5 months (range 1–9). In order to evaluate the response duration, all patients stopped FK therapy after a median of 5 months from CR (range 2–12). 2 pts relapsed after 2 and 1 patient after 3 months. All of these patients resumed FK therapy and re-achieved CR after 2 months. 1 patient lost CR but remained in PR (>40×9/L for >8 weeks) at 10 months follow-up; 1 patient showed a sustained CR (more than 20 months follow up). Cytokine levels were quantified by enzyme-linked immunosorbent assay (ELISA) technique with kits obtained from R&D Systems (Minneapolis, MN, USA). Serum γ IFN, IL-2 and s-IL-2R levels were also quantified. All samples showed IL-10 serum high levels (>500 pg/mL, range 7.8–500 pg/mL). Interesting, serum γ IFN and IL-2 were below defined cut-off (15 pg/ml and 1.2 U/ml) in all pts and serum levels IL-2 receptor were increased (cut-off 70 pg/ml). Our data suggest that IL-10 may be involved in limiting and terminating inflammatory responses in ITP patients. In this way IL-10 may be synergistic with FK anti-inflammatory property. FK, on the other hand, inhibits regulatory IL-10 effects on growth and/or differentiation of B cells.

**PO-110**

**THE ROLE OF RECOMBINANT ACTIVATED FACTOR VIIA IN A PATIENT WITH SEVERE THROMBOCYTOPENIA AND LIFE-THREATENING GASTROINTESTINAL BLEEDING**

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Severe thrombocytopenia is a common complication in hematologic malignancies, due to a decreased platelet production by bone marrow involvement or to the use of intensive chemotherapeutic regimens. Platelet transfusion is the current standard treatment for bleeding episodes associated with severe thrombocytopenia. However, the transfusion complications such as transfusion-transmitted diseases, platelet refractoriness, immunomodulation and difficulty to achieve a sufficient supply of platelets from donors, as well as the failure to achieve a proper hemostasis despite transfusion, prompt the search for therapeutic measures that may complement platelet transfusion. Recombinant activated factor VII (rFVIIa) has been shown to improve hemostasis in patients with thrombocytopenia in several studies. We report the case of a 58-year old man who was diagnosed as having primary thrombocythemia in 1974. The patient had been treated with chemotherapeutic agents, namely hydroxyurea, and later with interferon-α (INF-α) until he developed severe thrombocytopenia, mild anaemia and leukocytosis in 2001. The presence of bone marrow fibrosis, extramedullary hemopoiesis and splenomegaly suggested transformation to myelofibrosis with myeloid metaplasia. The patient was treated with supportive therapy until January 2003, when the myeloproliferative syndrome evolved into acute myeloid leukemia, with monosomy of chromosome 7. At that time, the patient also developed a thrombotic event (AMI with PTC on right
The patient was induced in complete hematologic and cytogenetic remission with FLAG regimen and, given the unavailability of a bone marrow donor, consolidated with intermediate doses of cytosine arabinoside. He relapsed after a disease free survival of 6 months as a chronic phase myelofibrosis and thus was treated with thalidomide, prednisone and androgens without achieving any response. Three months later, the patient presented with sudden precordial pain and dyspnea with severe anemia (Hb 4 g/dL) and thrombocytopenia (platelet: 6,000/mm³), associated with gastrointestinal bleeding without gross lesions at endoscopy repeated in multiple occasions. Endoscopic examination revealed only a diffuse bleeding from the gastric mucosa. A peripheral blood and bone marrow morphology examination showed high number of myeloid blast cells. He was transfused with platelets and red blood cells. However, bleeding continued despite an increase in the platelet count. Because the patient’s condition was deteriorating rapidly, intravenous boluses of rFVIIa at 100 μg/Kg were administered in concurrence with platelet transfusion every 3 hours for five doses. Bleeding stopped within six hours and allowed the patient’s condition to improve sufficiently to undergo a central venous catheter insertion, without hemorrhagic complications. Fifteen days later, the gastrointestinal bleeding relapsed with melena. Endoscopic examinations did not reveal gross lesions, whereas a diffuse gastric mucosal bleeding was reported. In concurrence with platelet transfusions, recombinant FVIIa was repeated at a dose of 100 μg/Kg every 3 hours for four doses, and then every 7 hours for ten additional doses with no further evidence of bleeding. Adverse effects of rFVIIa administration were not observed, despite the patient’s history of AMI. Overall, this case report suggests that rFVIIa administered in concurrence with platelet transfusion appears to be a valid alternative for controlling bleeding in patients with severe thrombocytopenia, especially when platelet transfusion alone are ineffective.

HHV8 is implicated in the etiopathogenesis of Kaposis sarcoma (KS) and rare lymphoproliferative disorders mainly occurring in HIV infected people. The occurrence of non-malignant disease such as bone marrow (BM) failure or hemophagocytic syndrome, in transplant recipients or HIV positive patients with KS, has also been linked with HHV8 infection in occasional cases. We report the occurrence of a fatal hemophagocytic syndrome in two HHV8 positive immunocompetent patients. Patient 1: a 63-year old woman was admitted with fever, peripheral cytopenia and splenomegaly. One month before she had been started on corticosteroid treatment for autoimmune haemolytic anaemia (AHA) and ten days before she had received nephrectomy for renal carcinoma. There was no evidence of metastatic disease and bacterial or viral infection. She received eritropoietin, G-CSF and high dose intravenous immune globulin, with no benefit and died with a rapidly evolving multiorgan failure. Patient 2: a 69-year old man was admitted for AHA and splenomegaly. He initially responded to corticosteroid treatment, but rapidly developed pancytopenia and fever. He received high dose intravenous immune globulin, acyclovir and broad spectrum antibiotics but died with rapidly progressive multiorgan failure. A BM aspirate or biopsy performed in all two patients showed a normo/hypocellular marrow with myelodisplastic features associated with signs of hemophagocytosis, without evidence of lyphomatous infiltration. HHV-8 DNA was detected either in the peripheral blood or in the serum from all two patients, by PCR for three different viral genes (orf-K1, the ORF 73 and orf 26). The molecular analysis also allowed us to determine the HHV-8 subtype which was variant A in patient 1 and variant C in patient 2. The occurrence of different viral genotypes in the two cases was also confirmed by the analysis of ORF 73 polymorphisms. The HHV-8 viral load was determined by real time PCR for orf 26, showing the presence of an extremely high number of viral copies in both cases. In patient 1 immunohistochemical analysis in the BM biopsy showed the presence of the HHV8-LNA positive cells. Pancytopenia with hemophagocytic syndrome, myelodisplastic features and AHA occurring in immunocompetent adults should be added to the spectrum of clinical pathologic manifestations associated with HHV8 infection. Its frequency may be underestimated and it should be always considered in the differential diagnosis of unexplained peripheral cytopenia. Its prompt recognition by molecular and immunohistochemical meth-
PO-113
A DESCRIPTION OF TWO CASES OF FACTOR V DEFICIENCY
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Congenital factor V deficiency is a coagulation disorder (1:1000000), usually transmitted as an autosomal recessive trait. Diagnosis is based on coagulation test showing prolonged activated partial thromboplastin time (aPTT), normal thrombin time (TT) and prothrombin time (PT) corrected by normal plasma adsorbed with aluminium hydroxide or barium salts. The patient 1, a 12-year old girl with thalassemia major, was admitted for bone marrow transplantation from a male sibling donor mismatched for one locus: on this occasion a heterozygous deficiency of FV (21%) was discovered. A study of the family members revealed that the mother also had severe deficiency (1%) but was asymptomatic and multiparous, whereas the father had normal levels (107%). The FV deficiency was not considered to be a contraindication for transplant, which was performed without the support of supernatant cryoprecipitate. The patient 2, a 14-year old girl, with a negative history until the age of 7, when she underwent surgery to remove a cutaneous preauricular appendix and FV deficiency (2%) was diagnosed. Menarche occurred at age 12, with regular menstruation of normal quantity and length. These two cases indicate that there is no correlation between the severity of the haemorrhagic symptoms and plasma levels of FV, since severe deficiencies are often silent, probably because the risk of bleeding depends more on levels of platelet FV, than to levels in the plasma. In these situations transfusion therapy may not be necessary, thus avoiding the well-documented risk associated.

PO-114
ENDOTHELIAL PROGENITOR CELLS ARE INCREASED IN THE PERIPHERAL BLOOD OF PATIENTS WITH MYELOFIBROSIS WITH MYELOID METAPLASIA

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Endothelial progenitor cells (EPCs) are identified by the co-expression of the CD34, VEGFR-2, and CD133 antigens on their surface and by their capacity to form colonies in vitro. It has been reported that these cells circulate at a very low extent in the peripheral blood (PB) of normal subjects. We have previously shown that patients with Myelofibrosis with myeloid metaplasia (MMM) have an increased number of circulating CD34+ cells compared to patients with other Ph-negative myeloproliferative disorders. We have also recently reported that the bone marrow and spleen of MMM patients are characterised by enhanced angiogenesis. In this study we have tested whether patients with MMM have a higher number of circulating EPCs compared to patients with Polycythemia Vera (PV) or Essential Thrombocytemia (ET) and to healthy subjects. Up to 50 ml of PB were obtained from patients and controls enrolled in the study and both cytofluorimetric studies and in vitro cultures were performed. For FACS analysis the following monoclonal antibodies were used: FITC anti-CD34, PE anti-CD133, and PerCp anti-VEGFR-2. Endothelial colonies were obtained by plating PB mononuclear cells in the presence of a commercial medium specific for endothelial cell growth. Colony identity was confirmed by in situ staining with monoclonal antibodies towards CD31, vWF, VE-cadherin, CD34, and CD45 and by staining with FITC-labeled Ulex europeus agglutinin. The median percentage of CD34+CD133+VEGFR-2+ cells in 58 patients with MMM was higher (1.2%, range 0-21.5) than in 11 patients with PV or ET (0%, 0-3.2) and than in 12
normal subjects (0%, 0–3.6) (p = 0.012, and p = 0.001, respectively). Analysis of CD34+ selected cells confirmed this observation. In fact, the percentage of CD34+ cells co-expressing the VEGFR-2 and the CD133 antigens was statistically higher in MMM patients (n = 12) than in normal subjects (n = 6) (p = 0.026). EPC cultures showed that the number of VE-cadherin+ CD34+ colonies in 16 MMM patients was higher (median 68, range 52–100) than in 8 PV/ET subjects (9, 4–16) and than in 6 healthy subjects (21, 5–37) (p < 0.05, and p < 0.02, respectively). However, in other 26 MMM patients the number of EPC colonies was in the range of those of controls. Taken together, our data suggest that in patients with MMM the circulating EPCs are increased compared to patients with other Ph-negative myeloproliferative disorders and to healthy subjects. This observation may be related to the enhanced angiogenic processes that characterize the disease.

**PO-115**

**ABL KINASE MUTATIONS DETECTED BY A NOVEL D-HPLC-BASED ASSAY IN LATE-CHRONIC PHASE CHRONIC MYELOID LEUKEMIA PATIENTS RESISTANT TO IMATINIB ARE STRONGLY ASSOCIATED TO SUBSEQUENT PROGRESSION TO BLAST CRISIS**

Soverini S,1 Rosti G,1 Amabile M,1 Poerio A,1 Bassi S,1 Trabacchi E,1 Giannini B,1 Ottaviani E,1 Renzulli M,1 Grafone T,1 Terragna C,1 Testoni N,1 Luatti S,1 Castagnetti F,1 Pane F,2 Izzo B,2 Fava M,3 Saglio G,3 Soverini S,1 Rosti G,1 Amabile M,1 Poerio A,1 Bassi S,1 Trabacchi E,1 Giannini B,1 Ottaviani E,1 Renzulli M,1 Grafone T,1 Terragna C,1 Testoni N,1 Luatti S,1 Castagnetti F,1 Pane F,2 Izzo B,2 Fava M,3 Saglio G,3 Alberti D,4 Baccarani M,1 Martinelli G1 on behalf of the GIMEMA CML Working Party

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**Background:** Mutations within the ABL kinase domain of the BCR-ABL gene are emerging as the most frequent mechanism of resistance in chronic myelogenous leukemia (CML) patients treated with Imatinib. Regular monitoring for emerging mutations should enter the clinical practice in order to assure a rational therapeutic management of these patients. **Aims:** aims of the present study were: a) to set up a novel, high-throughput and cost-effective method for screening of Imatinib resistance-associated mutations based on denaturing high-performance liquid chromatography (D-HPLC); b) to investigate the frequency, distribution and prognostic relevance of ABL mutations in 47 Imatinib-resistant CML patients enrolled in two multicenter trials of the GIMEMA CML Working Party. **Methods:** This study was performed on 86 bone marrow and/or peripheral blood samples from 47 patients showing upfront cytogenetic resistance to Imatinib. Seven patients were enrolled in the CML011/STI571 trial (newly diagnosed, early-CP CML patients treated with Imatinib 400 mg/d and pegilated-interferon) while the remaining 40 were enrolled in the CML002/STI571 trial (late-CP CML patients resistant/refractory to α-interferon, treated with Imatinib 400 mg/d). For each patient, a longitudinal mutational analysis was done on all the available samples collected from imatinib start to the twelfth month of therapy (or to treatment discontinuation for those patients who prematurely exited the protocols for progression or adverse events). A nested RT-PCR was set up and, for each sample, two partially overlapping fragments of 393 and 482 bp corresponding to ABL kinase domain were analysed in parallel by D-HPLC and sequencing. **Results:** in 22/47 (47%) patients, D-HPLC showed an abnormal elution profile suggesting a nucleotide change. Direct sequencing confirmed the presence of a mutation in all but three cases, where mutations were revealed only after cloning. Conversely, all the samples scored as wild-type by D-HPLC did not show evidence of mutations by sequencing. Two out of 7 (29%) resistant patients enrolled in the CML011 trial had mutations, thus confirming that the latter are rare in early-CP patients. Both mutations resulted in novel amino acid substitutions (F311I, E355D). Among resistant patients enrolled in the CML002 trial, 19/40 (45%) had mutations. Patients with missense mutations (n = 18) showed a significantly shorter time to progression to blast crisis (BC) (p = 0.0002) and overall survival (OS) (p = 0.001) when compared to patients with a wild-type ABL protein (n = 22). P-loop mutations were detected in 11 patients, of whom 9 subsequently progressed to BC and 6 died of their disease. By contrast, among the 7 patients without P-loop mutations, 2 progressed to BC and 1 died. **Conclusions:** In late-CP patients, presence of a point mutation was strongly associated with subsequent progression to BC; P-loop mutations seemed to confer a particularly poor outcome. D-HPLC proved to be a rapid, reliable and sensitive tool for large-scale mutational screening of patients undergoing Imatinib treatment.

**Funding:** Study supported by COFIN2003 (M. Baccarani), AIRC, AIL, Fondazione Del Monte di Bologna e Ravenna, FIRB2001.
The hypereosinophilic syndrome (HES) is a rare hematological disorder characterized by abnormal overproduction and accumulation of eosinophils causing organ damage. It can be distinguished the secondary forms, that can be caused by other causes such as parasitic infections, atopy, hypersensitivity reactions, collagen vascular disease, or tumors, and the idiopathic hypereosinophilic syndrome, for which diagnostic criteria have been proposed. These include: (1) sustained eosinophilia (1.5×10^9 cells/mmc) present for longer than 6 months, (2) absence of other known causes of eosinophilia, and (3) signs and symptoms of organ infiltration. HES occurs more frequently in men than in women, and the usual age of presentation is between 20 and 50 years. The organs and systems most frequently affected by HES include heart, nervous system, skin, lungs, liver, and gastrointestinal. Recently, a novel fusion tyrosine kinase, FIP1-like 1 (FIP1L1) gene to the PDGFR-a gene, has been found to be involved in some patients with idiopathic hypereosinophilic syndrome (HES) responsive to imatinib therapy, generated by an interstitial deletion on chromosome 4q12. We collected blood samples of 141 patients with hypereosinophilic syndrome, and we performed reverse transcriptase polymerase chain reaction (RT-PCR) analysis of FIP1L1-PDGFR-a. In our study 86 patients were affected by secondary hypereosinophilic syndrome (60%), 55 patients had idiopathic hypereosinophilic syndrome (40%). All the patients with secondary forms were negative for the presence of the FIP1L1-PDGFR-a rearrangement, whereas of the patients with the idiopathic forms 13 were positive. From December 2002 we treated 31 patients with idiopathic hypereosinophilic syndrome with different dosages of Gleevec (100-400 mg/die), 13 positive for FIP1L1-PDGFR-a, and 18 negative. 26 patients are male, five are female, with a median age of 52 years (range 20-78) and a peripheral blood count of 4,25×10^9 cells/mmc present for longer than 6 months, (2) absence of other known causes of eosinophilia, and (3) signs and symptoms of organ infiltration. HES occurs more frequently in men than in women, and the usual age of presentation is between 20 and 50 years. The organs and systems most frequently affected by HES include heart, nervous system, skin, lungs, liver, and gastrointestinal. Recently, a novel fusion tyrosine kinase, FIP1-like 1 (FIP1L1) gene to the PDGFR-a gene, has been found to be involved in some patients with idiopathic hypereosinophilic syndrome (HES) responsive to imatinib therapy, generated by an interstitial deletion on chromosome 4q12. We collected blood samples of 141 patients with hypereosinophilic syndrome, and we performed reverse transcriptase polymerase chain reaction (RT-PCR) analysis of FIP1L1-PDGFR-a. In our study 86 patients were affected by secondary hypereosinophilic syndrome (60%), 55 patients had idiopathic hypereosinophilic syndrome (40%). All the patients with secondary forms were negative for the presence of the FIP1L1-PDGFR-a rearrangement, whereas of the patients with the idiopathic forms 13 were positive. From December 2002 we treated 31 patients with idiopathic hypereosinophilic syndrome with different dosages of Gleevec (100-400 mg/die), 13 positive for FIP1L1-PDGFR-a, and 18 negative. 26 patients are male, five are female, with a median age of 52 years (range 20-78) and a peripheral blood count of 4,25×10^9 eosinophil cells /mmc (range 1.5-18) at diagnosis. In twelve patients we had a documented organ infiltration in different organs (lung, heart, skin, kidney). Cyto- genetic studies showed no evidence of either the bcr-abl translocation or TEL-PDGFRb, one patient had t(5;12)(q33;p13), one patient shown 45,x0, and one t(1,15). All the patients FIP1L1-PDGFR-a positive had a dramatically response to the therapy, with a decrease of the eosinophil number in the peripheral blood and in the bone marrow smears after seven days of therapy. No relevant toxicity was observed. In seven cases we studied the sequence of the fusion gene, mixing serially diluted total FIP1L1-PDGFR-a(e)+ RNA (diagnostic sample) with the HL60 cell line, and we were able to amplify the transcript up to a 1:10(e)4 dilution. Sequence analysis was performed to confirm which is the breakpoints in PDGFR-a and if the different bands represent splice variants.

**Funding:** Supported by: COFIN 2003, by FIRB 2001, by the University of Bologna (60% grants), by the Ital-
CLINICAL-HEMATOLOGICAL FINDINGS AND MOLECULAR RESULTS IN A NEW SERIES OF CHRONIC EOSINOPHILIC LEUKEMIA WITH 4q12 CRYPTIC DELETION

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According to WHO criteria, chronic eosinophilic leukemia (CEL) represent myeloproliferative disorders with increased eosinophils in the peripheral blood (>1.500/mL) lasting more than six months, exclusion of secondary causes, presence of signs or symptoms of organ involvement, and demonstration of clonality of eosinophils or increased bone marrow blasts. We report on clinical-hematological and molecular findings in ten patients with 4q12-/CEL. Cytogenetics was done on bone marrow cells after culturing 24/48 hours. Metaphases were G-banded with Wright stain and karyotypes were described according to the International System for Human Cytogenetic Nomenclature (1995). Interphase-FISH was performed with a set of probes, RP11-120K16, RP11-3H20 (kindly provided by Dr P Marynen, University of Leuven, Belgium), designed to detect 4q12 cryptic deletion which underlies the FIP1L1/PDGFRα rearrangement. In three patients PCR investigations confirmed the FIP1L1/PDGFRα fusion protein. Patients were nine males and one female aged between 29 and 68. All patients had an hyperleukocytosis (7000-129000/L) with increased eosinophils (range of absolute number: 2088-30048). Increased bone marrow eosinophils were documented in 5/6 patients with available bone marrow biopsy. Splenomegaly was present in 8/10 while hepatomegaly was present in all 7 analysed cases. Other signs or symptoms of organ involvement were present in 7/10 patients with the following distribution: heart 3 cases, skin 3 cases, lung 2 cases, central nervous system 2 cases. Karyotypes were normal in 7/10 analysed patients. The cells bearing deletion of clone RP11-3H20 ranged from 43% to 96% at diagnosis. Treatment with imatinib mesylate induced rapid hematological remission in 7/7 patients. FISH and PCR are compared in the monitoring of molecular remission.

ANTHI CD-20 TREATMENT HALTED PROGRESSION OF THE HAEMOLYTIC PROCESS IN MYELOFIBROSIS WITH MYELOID METAPLASIA PATIENT (MMM) AND SEVERE TRANSFUSION DEPENDENT ANEMIA. A CASE REPORT

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Some reports suggest that immunologic mechanism may play a role in the pathogenesis of anemia in myelofibrosis with myeloid metaplasia (MMM). Moreover, rituximab, a chimeric monoclonal antibody against CD-20, has been increasingly recognised as a useful therapeutic agent for immuno-mediated disorders. We report of a patient suffering from MMM and severe transfusion dependent anemia in whom anti CD-20 treatment halted progression of the haemolytic process. A 70-year-old male was diagnosed on January 1997 as MMM low risk, pathologic stage II with normal karyotype. Diabetes and paroxysmal atrial fibrillation were co-morbidities. Because of thrombocytosis and abdominal discomfort due to liver and spleen enlargement, hydrea was given at dose of 20 mg/Kg three times weekly. Treatment with danazol 200 mg/daily, rhEPO 10000 U scx3 weekly and folic acid was added 5 years later, when Hb level dropped below 90 g/L. Nevertheless a median of 2 packaged red blood cell units was needed monthly. Increased serum levels of unconjugated bilirubin and LDH, a slightly reduction in serum haptoglobin and C3c along with a mild reticulocytosis, were suggestive of hemolytic anemia, despite DAT negative test (IgG, IgA, C3d), CD55, CD59 normal flow cytometric assay, normal GP6D level and absence of cold agglutinins in the plasma. According to published data concerning the cyclosporine-A effects in improving anemia in MMM, a cyclophosphamide dose of 100 mg/daily was performed. The patient became transfusion free in 5 months and he did not need any transfusion for one year long. At the time of relapse, EDX was stopped and after three months of azatioprine treatment, anemia progressively worsened and the patient became heavily transfusion dependent. Keeping in mind his clinical history, we decided to treat the patient with rituximab to inhibit the underlying immunologic mechanism. Rituximab was given in a 500 mg total dose, once weekly on 4 consecutive times. Response to treatment was evaluated by the improvement of the parameters of hemolysis, such as decrease in bilirubin and LDH, increase in Hb levels.
and of course, as reduction in transfusion need. Three weeks after the last rituximab infusion, Hb level rapidly improved, reaching 11 g/L, and the patient did not need any blood transfusion for the following three months. Stricking, bilirubin and LDH progressively decreased about of 50%. An immunomediated mechanism negatively affects erythropoiesis in MMM. Furthermore a negative Coomb’s test does not exclude autoimmune hemolytic anemia in such of patient. Rituximab is a chimeric monoclonal antibody directed against normal and malignant mature B-lymphocytes and results in prolonged and severe B-cell depletion. By this mechanism Rituximab inhibits autoimmune response against erythroid progenitor cells and/or circulating red cells. Rituximab may also interfere with cross-talk between T and B cells in blocking the release of cytokines known to impair erythropoiesis, eg. TNFα and IFNγ. Case reports on the use of rituximab in MMM patient with immune-haemolytic anemia are very scant. Our results suggest that this such of approach is very encouraging, but further investigations are warranted.

PO-119
SUSTAINED COMPLETE MOLECULAR REMISSION AFTER DISCONTINUATION OF IMATINIB THERAPY IN CML PATIENTS
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Imatinib mesylate (IM) therapy leads complete cytogenetic response (CCyR) in the majority of patients with chronic myelogenous leukemia (CML) in chronic phase. A few pts achieve complete molecular remission (undetectable BCR-ABL by Q-PCR). Aim of this study was to describe the clinical outcome of four patients (see Table below) who discontinued IM therapy after obtaining a complete molecular remission in bone marrow and peripheral blood. The relative quantification of BCR-ABL transcript was performed by real-time PCR using SybrGreen I as double-stranded DNA binding fluorescent dye. Both forms of p210 BCR-ABL transcript (b2a2 and b3a2) were detectable with the same set of oligonucleotides by analysing dissociation curves. A serial dilution of total RNA from K562 cells was used to construct a standard curve for real-time quantification. The sensitivity threshold for BCR-ABL mRNA quantification was fixed at 10⁻⁴ dilution standard mRNA, corresponding to 6pgs of RNA. BCR-ABL expression levels were normalized to ABL mRNA expression and calibrated on K562. The 4 pts were interferon pre-treated with a long history of disease. One pt had obtained a CCyR on IFN and crossed to Imatinib because of IFN-intolerance, the other 3 were IFN-refractory. None of them had a familiar donor for allotransplant or had an indication for unrelated transplant. While on IM therapy at 400 mg/daily, no patient required dose reduction or suspension due to hematologic or non-hematologic toxicity. All obtained CCyR at month 6. Negativity of Q-PCR was documented at month 9 in two pts and at month 12 in the other two. At the time of IM withdrawal, patients had been in continuous complete molecular remission for a period ranging from 13 to 19 months and showed normal morphology and negative cytogenetics at bone marrow examination. Reasons for IM withdrawal were patient’s refusal of treatment in one, suboptimal adherence to the prescribed dose in the other 3. Patients are still monitored by Q-PCR every 3 months on peripheral blood and bone marrow. All 4 patients are currently Q-PCR negative after 6 to 8 months from IM discontinuation. Despite the absence of detectable disease by Q-PCR, these pts may have subclinical quiescent Ph'-positive stem cells that can be a source for disease relapse; but is also possible that control of minimal residual disease may occur by the normal marrow. However, although the follow-up is relatively short, these data show that in selected patients with complete sustained molecular response to Imatinib, unmaintained molecular remission may persist after drug withdrawal.

| Gender/ Sokal Date Hemat Cy Resp Time to Time to Time from Diag CCyR Q-PCR Q-PCR neg IM withdrawal |
|--------------|---------|-----|--------|-----------|-----------|-----------|-----------|-----------|
| 1 M/60 low 1990 yes NR 137 6 9 13 8 |
| 2 F/51 high 1999 yes CCyR 33 -- 12 17 7 |
| 3 F/52 int 1995 yes NR 66 6 12 14 7 |
| 4 M/63 low 1998 yes NR 33 6 9 18 6 |

All timing measures are in months.

PO-120
CO-TREATMENT WITH DIFFERENT HDAC INHIBITORS AND IMATINIB MESYLATE RESTORES STI SENSITIVITY IN RESISTANT BCR/ABL POSITIVE CELLS
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Imatinib mesylate (STI571, Gleevec) is a powerful inhibitor of the tyrosine kinase activity of Bcr/Abl, the oncprotein responsible for chronic myeloid leukemia (CML). Histone deacetylase inhibitors (HDIs), short
chain fatty acids such as sodium butyrate, suberoylanilide hydroxamic acid (SAHA), and valproic acid, represent a novel class of agents that act by promoting histone acetylation. This in turn leads to transcriptional activation of diverse genes. HDIs have been shown to induce maturation and apoptosis in various human leukemia cells. Recently, SAHA has been demonstrated to enhance STI571 induced apoptosis in CML cells. We evaluated the effect of combination of Imatinib with several HDAC inhibitors, a stable prodrug xylitol derivative of butyrate (D1), valproic acid and SAHA on Bcr/Abl positive cell lines, LAMA84 and K562, and on LAMA84 resistant subline, LAMA84RR 0.6 and 8 CML-blastic crisis resistant to STI primary cells. The anti-proliferative effects of HDIs as single agents and in combination with STI were analyzed. Both LAMA and K562 cell growth was inhibited by D1, with an IC50 of about 0.5 mM and to valproic acid with an IC50 of 0.5 microM, respectively. On the contrary, LAMA84Ro.6 showed a cross resistance to both D1 and valproic acid, with IC50 >2 mM and >2 µM. Co-treatment with STI571 (0.2-1.0 µM) and D1 (0.5-1.0 mM) strongly inhibited cell proliferation, resulting in a marked synergistic effect in sensitive. In resistant subline, the ID50 was significantly modified. In LAMA84 and LAMA84R inhibition of cell growth was paralleled by induction of apoptosis, as demonstrated by Annexin V positivity and caspase-3 cleavage, as well as by cell cycle analysis. Interestingly, Imatinib as single agent induced a blockade of cell cycle in G0/G1 phase, exerting a cytostatic rather than a cytotoxic effect, whereas co-exposure to Imatinib and butyrates resulted in a marked activation of apoptotic pathways. We further evaluated the effect of a sequential scheduled treatment. LAMA84 and LAMA84R cells were exposed to HDIs for 24 hours, then thoroughly washed and incubated with Imatinib for 48 hours. This drug scheduling induces apoptosis in Bcr/Abl+cell lines, more potently than simultaneous exposure to butyrate and imatinib. Restoration of STI sensitivity in LAMA84R cells was demonstrated only after simultaneous treatment with HDIs and imatinib as well as after the sequential scheduled treatment. These findings show that Imatinib/butyrate combination may overcome in vitro resistance to Imatinib, and suggest that the strategy of combining STI571 with histone deacetylase inhibitors warrants consideration in CML and related hematologic malignancies. We also evaluated by Western Blotting the role of P-Tyr and Hsp90, a molecular chaperone involved in signal transduction, cell cycle regulation and cell survival. The fusion protein Bcr/Abl could be subject to ubiquination and proteosomal degradation in absence of Hsp90.

The hallmark of chronic myeloid leukemia (CML) is the chromosomal translocation t (9; 22)(q34; q11.2). In most cases, the resulting fusion genes give rise to different length products corresponding to chimeric BCR-ABL proteins of 210 or, more rarely, 190 or 230 kDa, respectively. We report the case of a patients diagnosed as having CML, showing atypical hematologic features and a very uncommon BCR-ABL rearrangement. A 45-year old woman, only son, was referred to our attention because of a normochromic anemia, thrombocytosis and a mild basophilia, discovered through a routine blood count. On admission, Hb was 11.7g/dl, Hct 33.1%, white blood cells 4,650/mL (neutrophils 1,870/mL, lymphocytes 2,440/mL, monocytes 190/mL, eosinophils 69/mL and basophils 280/mL) and platelets 531,000/mL. A comprehensive laboratory work-up showed no further abnormalities. Physical and imaging examinations revealed no pathological findings; no spleen or liver enlargements were found. The peripheral blood smear confirmed the thrombocytosis and the mild increase in the number of basophils; some hypogranulated and banded neutrophils were also noted. The BM was markedly hypercellular mainly because of a granulocytic and megakaryocytic hyperplasia. Most megakaryocytes were monolobulated. Myelodisplastic features, such as hyposegmented and hypogranulated granulocytes, were also found. The G-banded karyotype revealed a typical t (9; 22) chromosomal translocation in 5 of the 25 (20%) metaphases analyzed. Reverse transcription-polymerase chain reaction (RT-PCR) analysis, using primers derived from bcr exon e1 and abl a3, showed an atypical amplification band. Sequencing analysis of the PCR product revealed that the breakpoint on the BCR gene fell within exon 8. Moreover, the portion of exon e8 of the BCR gene was not directly joined to ABL exon a2 due to an insertion of 15bp derived from ABL intron b1, giving rise to an in-frame e8-int-a2 BCR-ABL fusion transcript. This latter was translated into a p200 BCR-ABL protein. Further molecular analyses excluded the activation of the same genes, which are
PO-122
IMATINIB IN LATE CHRONIC PHASE CML PATIENTS.
LONG-TERM MOLECULAR EVALUATION IN COMPLETE CYTOGENETIC RESPONDERS

Bassi S,1 Amabile M,1 Martinelli G,1 Rosti G,1 Trabacchi E,1 Castagnetti F,1 Giannini B,1 Cillonii D,2 Izzo B,3 De Vivo A,1 Testoni N,1 Rege Cambrin G,2 Soverini S,1 Luatti S,1 Iacobucci I,1 Gottardi E,2 Bassi S,1 Amabile M,1 Martinelli G,1 Rosti G,1 Trabacchi E,1 Castagnetti F,1 Giannini B,1 Cillonii D,2 Izzo B,3 De Vivo A,1 Testoni N,1 Rege Cambrin G,2 Soverini S,1 Luatti S,1 Iacobucci I,1 Gottardi E,2 Bassi S,1 Amabile M,1 Martinelli G,1 Rosti G,1 Trabacchi E,1 Castagnetti F,1 Giannini B,1 Cillonii D,2 Izzo B,3 De Vivo A,1 Testoni N,1 Rege Cambrin G,2 Soverini S,1 Luatti S,1 Iacobucci I,1 Gottardi E,2 Dorigo A,4 Pane F,3 Salvatore F,3 Saglio G,2 Baccarani M1 (on behalf of the GIMEMA CML Working Party)

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Imatinib (Ima) is a tyrosine-kinase inhibitor, highly effective in CML. The proper follow-up of Ima treated patients is based on cytogenetic (conventional and FISH, as appropriated) and molecular techniques: particularly, complete cytogenetic responders (CCgR) require the assessment of molecular response (MR) through a molecular quantification. The long-term molecular follow up of these patients would allow to evaluate the rate of overall and major molecular response and the prognostic impact of different levels of bcr-abl transcript reduction, given the same, complete, cytogenetic result. The GIMEMA CML Working Party conducted a phase II clinical trial (CML002/STI571) in late chronic phase CML patients, resistant to or intolerant of recombinant interferon. During this study, the cytogenetic response was assessed after 3, 6, 12 months on Ima and every 6 months thereafter. The MR was evaluated (bone marrow) at the same check-points. MR was assessed with Real-time quantitative (Taqman) RT-PCR and was expressed as the ratio between BCR/ABL and β2-microglobulin×100. The lowest level of detectability of the method is 0.00001 (major molecular response).

Since August 2000 284 patients were enrolled and treated with Ima 400 mg/daily. One-hundred and fifty-one out of 284 (53%) obtained a CCgR; 114/151 (75%) obtained the CCgR within 12 months on Ima. Ninety-one/114 patients (80% of CCgR responders or 32% of the whole patient population) maintained the CCgR in the long-term (stable CCgR). The results on the CCgR of these CCgR have been published (Blood 2004; 103:2284–2290): the median value of MR was 0.0008 after 12 months and 0.0001 after 24 months, the transcript level being undetectable in 22 cases. A reduction of the transcript level of more than 2 logs was achieved in all but nine cases of CCgR vs none of 23 cases of partial cytogenetic responders. These results, with a median follow-up of 36 months, will be updated.

Funding: Study supported by COFIN2003 (M. Baccarani), AIRC, AIL, Fondazione Del Monte di Bologna e Ravenna, FIRB2001.

PO-123
ROLE OF CIRCULATING CD34+ CELLS IN THE DIAGNOSIS AND FOLLOW-UP OF PATIENTS WITH CHRONIC MYELOPROLIFERATIVE DISORDERS

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Recently, our group (Passamonti et al., Haematologica 2003) demonstrated that an increased number of circulating CD34+ cells is an integral feature of myelofibrosis with myeloid metaplasia (MMM). To substantiate these results, we expanded previous data. In a prospective study, we evaluated the clinical utility of this parameter in 298 consecutive patients with chronic myeloproliferative disorders (CMDs). These included 121 patients with polycythemia vera (PV), 113 with essential thrombocytemia and 64 with myelofibrosis with myeloid metaplasia (MMM). The patients were followed-up for a median of 54 months (range 12–144 months). MM patients showed a significantly higher mean circulating CD34+ cell count in comparison with PV (p < 0.0001) and essential thrombocytemia (p = 0.0002). Similarly, a significant correlation was found between circulating CD34+ cell count and white blood cell (WBC) count, platelet counts, plasma erythropoietin (EPO) levels, splenic volume and digital capillaroscopy score (p < 0.0001 in all cases). The separation of patients into risk groups based on circulating cell count was able to predict survival in MMM (p < 0.0001).

Recently, we demonstrated that the circulating CD34+ cell count (median 1.5% of the white cell count) is a useful parameter to predict the response of patients with MMM to the JAK2 inhibitor ruxolitinib. The circulating CD34+ cell count is an integral feature of the disease, and is highly correlated with other manifestations of MM, such as splenomegaly, leukocytosis and thrombocytosis. A decrease in the circulating CD34+ cell count is an early sign of a response to ruxolitinib.
cytopenia (ET), and 64 with MMM. We prospectively followed 103 with sequential (yearly) enumeration of circulating CD34+. The median number of circulating CD34+ cells was 2.3×10^6/L (range 0–5) in 20 control subjects, 2.3×10^6/L (range 0–14) in PV, 2.4×10^6/L (range 0–14) in ET, and 96×10^6/L (range 4–4620) in MMM. We confirmed on a larger number of patients that the best cut-off value for distinguishing between MMM and PV or ET is 15×10^6/L, a threshold that can be reliably employed in the differential diagnosis of CMD. The sequential evaluation of CD34+ cells showed that MMM evolving from PV or ET is associated with elevated circulating CD34+ cell counts. In conclusion, enumeration of circulating CD34+ cells should be included in the workup of patients with CMD.
mg/day (Haematologica 2002;87: N Engl J Med 2001;344). We analyzed the hematological and cytogenetic response in a small cohort of patients who received imatinib at dose < 350 mg/day. We treated 20 patients at < 350 mg/day because of side effects or elevated age. The patients were followed up with physical examination complete blood count and differential weekly for the first month, every 15 days for the second month and monthly thereafter; renal and liver parameters were checked monthly. A bone marrow aspiration was performed every 3 to 6 months after the start of therapy. Of the 20 patients, 13 received the standard dose of 400 mg/day for a mean of 54.3 days and reduced the dose thereafter because of side effects (persistent leukopenia and thrombocytopenia intense leg pain often with reduced motility requiring non-steroidal anti-inflammatory drugs in absence of previous history of arthritis). Six patients were started with 300 mg/day because of age or cardiac failure. The Sokal risk stratification in this group of patients was: 14 patients in low risk, 4 in intermediate risk and 2 patients in high risk. All patients received a mean dose of 229,411 mg/day with a mean follow-up of 11,9 months (range 9,2–33,6). All patients started with the lower dose achieved hematological remission in a mean of 18,5 days; those who reduced the dose for side effects remained in complete hematological remission. 19/20 patients showed a major cytogenetic response after 3–6 months of therapy. During the follow up, in two patients a dose escalation was needed after 6 months, because of cytogenetic relapse; one patient stopped treatment because of hepatic failure; 8 patients remain in hematological and cytogenetic response, but not in molecular response; 9 patients, indeed, has showed molecular response too, with marked decrease in p210 RNA copies. These data suggest that even with reduced doses imatinib may give excellent responses in selected CML patients.

**PO-126**

**EMERGENCE OF TRISOMY 8 IN PH-NEGATIVE CELLS DURING IMATINIB MESYLATED THERAPY IN A CML PATIENT: A CASE REPORT AND REVIEW OF THE LITERATURE**

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The recent introduction of imatinib mesylate, the novel Abl–specific kinase inhibitor, represents a very important therapeutic option in chronic myelogenous leukemia (CML), inducing complete cytogenetic response (CCR) or major cytogenetic response (MCR) in a considerable number of patients in all CML phases. In a few CML patients who obtained a cytogenetic response, the development of additional chromosomal aberrations in Ph-negative cells during imatinib mesylate treatment has been recently described. The most common cytogenetic abnormality observed was trisomy 8. We report a case of an old CML patient, pretreated only with hydroxyurea, who was administered imatinib for disease progression to accelerated phase. The metaphase karyotypic analysis performed after 6 and 12 months of imatinib therapy revealed the appearance (in MCR) and persistence (in CCR) of a new aberrant Ph-negative clone with trisomy 8. Morphological analysis of bone marrow performed after 6 and 12 months of therapy, showed atypical megakaryocytic hyperplasia, mild dysplastic features of myelopoiesis, dysplastic erythropoiesis. Twenty-four months later the patient is still in CHR (complete hematological response). The biological, clinical and prognostic significance of this phenomenon is still in debate: although the development of trisomy 8 in Ph-negative cells seems not represent a potential adverse event, however the reports are still limited and the follow-up is too short. We welcome further studies and a case register for the evaluation of long-term significance of this abnormality and moreover we recommend a close monitoring of all CML patients on imatinib by classical cytogenetic analysis even after achieving CCR.

**PO-127**

**DETECTION OF FIP1L1-PDGFR FUSION GENE IN ELEVEN PATIENTS WITH HYPEREOSINOPHILIA**


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Chronic eosinophilic leukemia (CEL) and idiopathic hypereosinophilic syndrome (IHES) are rare hematological disorders showing unexplained and persistent peripheral blood hypereosinophilia due to eosinophil overproduction by the bone marrow. Their clinical course may be indolent or aggressive with functional damages targeting various peripheral organs. The World Health Organization (WHO) has established accurate diagnostic criteria for CEL and IHES: presence of hypereosinophilia (>1.5×10⁹/L) per-
sisting for more than 6 months with signs and symptoms of organ involvement, absence of causes of reactive eosinophilia (parasitic infections, allergic reactions). In addition, CEL is diagnosed in patients with proven clonality (patients either with a cytogenetic or a molecular defect), in contrast IHES in all the other cases. Recently, a novel fusion gene, FIP1L1-PDGFRA, resulting from an 800 kb interstitial cryptic deletion on chromosome 4q12, has been identified as the recurrent clonal molecular abnormality in patients with CEL. The FIP1L1-PDGFRA chimeric gene, which may be identified by in situ hybridization (FISH) or by nested RT PCR, produces a constitutively active tyrosine kinase, strongly inhibited by imatinib (STI-571).

In the present study a RT PCR assay was used for detecting the chimeric transcript FIP1L1-PDGFRα in twelve patients who fulfilled the WHO criteria for IHES and CEL. In particular nine patients were males and three females; their median age was 51.5 (22-70). All patients had been submitted to cytogenetic analysis which showed a completely normal chromosome pattern. In addition, FISH with probes specific for the chromosome breakpoints most frequently involved in hypereosinophilic syndromes but not for FIP1L1 and PDGFRα was also applied. It always showed a normal pattern. As far as RT PCR is concerned, total RNA was extracted from peripheral blood or bone marrow mononuclear cells, using RNeasy kit (Qiagen) and reverse transcribed with random hexamers and iScript (Invitrogen) according to manufacturers conditions. Nested PCR amplification was performed with the following primers: FIP1L-F1 ACCGCGGTGGATCTCTTGAT and PDGFRα-R1 TGAGAGCTGTGGTTTACTGGA; FIP1L-F2 AAAGAGGATACGAAATGGGACTTG and PDGFRα-R2 GGGACCGGCTTAATCCATAG, into a final reaction volume of 50 ml. PCR products were analysed by 2.5% agarose gel electrophoresis. A FIP1L1-PDGFRα chimeric transcript was detected in three out of the twelve patients. Therefore, basing on WHO criteria the three FIP1L1-PDGFRα positive patients were classified as CEL, whereas all the other 8 were considered IHES. The former patients were included in the Italian Cooperative Study on Idiopathic Hypereosinophilic Syndrome (IRES) and underwent treatment with escalating doses of imatinib. Two patients were monitored with qualitative RT PCR, achieved negative results and therefore entered a molecular and hematological complete remission (CR). In conclusion our results confirm that FIP1L1-PDGFRα positive CEL must be considered a rare well-defined clinicobiological entity, highly responsive to imatinib.

**PO-128**

**A CASE OF PHILADELPHIA NEGATIVE CHRONIC MYELOID LEUKEMIA WITH THE ABL/BCR FUSION GENE ON CHROMOSOME 9**

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Chronic myeloid leukemia (CML) is probably the most extensively studied human malignancy. The molecular hallmark of CML is the formation of a bcr-abl fusion gene, usually formed as a consequence of the Philadelphia (Ph) translocation involving chromosomes 9 and 22. Approximately 5% of patients with CML do not reveal the Ph chromosome cytogenetically and are termed Ph-negative CML cases. We report the case of a patient, admitted to another institution, who was made a diagnosis of Ph-positive CML. Initially, the patient received administration of interferon having a severe reaction then a daily oral administration of Imatinib mesylate (formerly STI571: Glivec TM) was started at 400 mg daily, but the drug gave all sorts of unpleasant symptoms. A hydroxyurea therapy was defined. Of late the patient referred to our clinic for a second opinion and treatment. Cytogenetic analysis showed a normal karyotype whereas the reverse transcriptase polymerase chain reaction technique revealed a rearrangement of the M-bcr and expression of a chimeric bcr/abl mRNA of b2a2 configuration. We have performed fluorescence in situ hybridization (FISH) analysis using a commercially available dual color, dual fusion, bcr-abl translocation probe (Vysis), which revealed atypical signal patterns in all mitoses observed: the presence of the bcr-abl fusion was found to be localized to chromosome 9 long arm, an abl probe signal was observed on the other chromosome 9 and two bcr signals of different sizes on each of chromosomes 22. Other cases of a fusion abl and bcr gene localized to chromosome 9q34, and not on chromosome 22q11, have been documented in the literature, and the clinical data reported a poor therapeutic response and outcome in such patients, suggesting a possible worse prognostic impact of this aberrant location of the bcr-abl. We decided to start therapy with Glivec at daily doses of 100 mg considerably lower than those dosages required for the treatment of CML and
no severe reaction were observed. After six months of therapy, our patient is in haematologic remission; \textit{in situ} hybridization detected the \texttt{bcr-}abl fusion to be located on both chromosomes 9 in 10% of the metaphases examined, while the remaining cells showed co-localization at only one of the chromosomes 9. In despite of fact that the patient showed no cytogenetic response, the haematologic remission is probably due to outstanding efficacy of Glivec that binds to a CML chimeric product leading the binding site in the catalytic center of the kinase in consideration of proved growth inhibitory effects. In the present study, we demonstrated that FISH analysis plays an increasing role in the diagnosis and monitoring therapeutic response of CML patients with unusual Ph translocation; in fact, if \texttt{M-bcr} specific primers are used in such patients, the expression of a typical \texttt{bcr-}abl mRNA will be obtained, precluding the possibility to define a subgroup of patients with different clinical and prognostic outcome compared to their \texttt{bcr-}abl-positive counterparts. Furthermore, since a favourable clinical course is confirmed in this case of tolerance of Glivec at low doses, we suggest that the routine use of FISH analysis might have an impact on the choice of treatment.

**PO-129**

**INTRASPLENIC CHEMOTHERAPY IN PATIENTS WITH CHRONIC MYELOPROLIFERATIVE DISORDERS AND HYPERSPLENISM**

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Chronic myeloproliferative disorders (CMD) may cause severe hypersplenism with subsequent cytopenia and abdominal pain. When conventional therapy fails, splenectomy or splenic irradiation remain the only therapeutic option for hypersplenism. \textit{Intra-arterial} chemotherapy through a percutaneous arterial catheter, which is often used for the treatment of solid tumors, is not commonly used in malignant hematologic diseases. In our study we performed intrasplenic infusion of cytosine arabinoside (\texttt{ARA-C}) in patients with CMD in which conventional therapy had failed or had been too toxic, to improve the blood cell count and reduce the volume of spleen and the abdominal pain. We treated 3 patients, 2 female and 1 male, median age 65 years (range 53–77); 2 of them had myelofibrosis with myeloid metaplasia (MMM), 1 had Philadelphia negative chronic myeloid leukemia (Ph–CML). A Port-a-cath was implanted at the upper third of the right thigh. The right femoral artery was punctured and the splenic artery was selectively catheterised. Intrasplenic \texttt{ARA-C} was administered at the dose of 10mg/m²/day for 5 consecutive days/month, continuous infusion. The dosage was increased each month by 10 mg/m²/day up to a maximum of 50 mg/m²/day for 5 consecutive days/month. We observed in all patients reduction of the spleen volume (4 cm/day reduction on average) and improvement of the abdominal pain and of the anemia (1.5 g/dl increase on average). Two of the three patients are alive (12 and 8 months after the beginning of intrasplenic chemotherapy), one underwent splenectomy two months after the beginning of intrasplenic chemotherapy and died two months after a MUD BMT. We did not observe any hematologic toxicity. Local haemorrhage occurred in two of the three patients when the PAC was implanted. In CMD with hypersplenism, with pain and cytopenia, an intrasplenic chemotherapy may be useful. This procedure reduces the abdominal pain and the spleen volume and slightly improves the blood cell count without showing haematologic toxicity. It is very important to check carefully the platelet count and clotting tests when the PAC is implanted to avoid local haemorrhages.

**PO-130**

**BONE MARROW NECROSIS DURING TREATMENT WITH IMATINIB MESYLATE FOR A BLASTIC CRISIS OF CHRONIC MYELOID LEUKEMIA**

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Bone marrow necrosis is a rare event that occurs after chemotherapy for acute leukemia or other malignant disease. We describe a case of a 67-year old man affected by chronic myeloid leukemia, BCR-ABL positive, started with a myeloid blastic crisis. The patient performance status was excellent and the only clinical sign of the disease was splenomegaly (longitudinal diameter 20 cm). At the diagnosis we found: high leukocytic count (WBC 190.000 mm³ with 70% of myeloid blast), mild anemia (HB 10 gr/dl), normal platelet count. Hepatic and renal test were normal. The patient began treatment with 600 ml/die of imatinib mesilato associated for the first week with hydroxyurea 2gr/die. The treatment induced a rapid disappearance of blasts. After 20 days the patients developed a severe aplasia that required supportive treatment with red cells and platelet transfusion, administration of G-CSF. Treatment with imatinib mesilato was stopped. Together with aplasia the patient reported an intense pain at the left leg that increased during walking. A bone scanning with tecnetium did not evidence abnormal accumulation.
T1 weighted magnetic resonance imaging scanning of the legs showed, in both femours, diffused round lesions with a low signal intensity without significant contrast impregnation in T2 weighted imaging: this was indicative of bone marrow necrosis. Usually it is asymptomatic; it develops in large bones (femours and pelvis) and its extent is directly related to the disease stage. This is the first report of bone marrow necrosis occurring during treatment with imatinib mesilato at standard dosage. Therefore our experience suggests the need of special care when treating for the first time with imatinib mesilato patients affected by advanced stage of chronic myeloid leukemia, in order to rapidly detect complications related with a too rapid blasts apoptosis.

**PO-131**

Cytogenetic resistance and hematological sensitivity to imatinib therapy in CML: the role of apoptosis related gene

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The introduction of Imatinib has greatly modified the picture of CML significantly increasing the number of complete remissions that were very few with previous therapies. Imatinib mesylate has been shown to powerfully and selectively inhibit ABL protein tyrosine kinase activity and to suppress in vitro and in vivo growth of BCR-ABL positive cell lines. It seems that escape from apoptosis may be a requisite event in the development of malignant neoplasms. Genes that can influence cell viability versus cell death have been described, including genes belonging to the bcl-2 family. Related genes of this large family encode proteins that regulate apoptosis both in a negative and, in some instances, positive fashion. In addition, alterations in the expression of these genes may cause aberrations in cell death and thus contribute to cancer. We evaluated, by reverse transcriptase polymerase chain reaction (RT-PCR), the expression of genes related to apoptosis, such as bcl-2, bax, bcl-X, and survivin, in cytogenetic resistant, but hematological sensitive to Imatinib patients. We compared such results with the expression of apoptosis related proteins in blast phase of CML patients and in diagnosis samples. We studied 10 patients, enrolled in the Novartis-sponsored multi-institutional Phase II trials. All these patients after 24 months of Imatinib therapy were in maintained complete hematological response while all analyzed metaphases showed the persistance of Ph chromosome. The data obtained from these patients were compared with those obtained from the study of 5 diagnosis sample, 5 cytogenetic responders to Imatinib therapy and 2 myeloid and 2 lymphoid blastic phases. (i) Bcl2 expression: bcl2 gene expression was found in 10/10 cytogenetic resistant patients and in 5/5 diagnosis samples. Bcl2 expression was absent in cytogenetic responders and in blastic phase patients. (ii) Survivin expression: survivin gene expression was found to be positive in the 24 cases studied. (iii) x- Bax expression: bax gene expression was found to be positive in all the haematological responders. Among the diagnosis sample 3 cases disclosed the gene expression. Two high risk patients do not show bax gene expression. The 2 myeloid blastic crisis samples lost gene expression while in 2 lymphoid blastic crisis the expression was still detectable. (iv) Bcl-X expression: In all the blastic crisis and in the diagnosis samples the gene expression was not present. The 10 cytogenetic resistant cases studied disclosed the presence of the expression with the preponderance of the antiapoptotic isoform. The complete cytogenetic samples disclosed the absence of gene expression (two cases) or the prevalence of the proapoptotic isoform. It is thus possible that in those patients who achieve haematological but not genetic remission due to specific tyrosine-kinase inhibitor, can occur, both a block in proliferation and an immediate reactivation which should be mediated by a pathways involved in the immortalization of the neoplastic clone. The understanding of these processes may be of help in determining the pathogenesis of this diseases and in the therapeutically trials.

**PO-132**

Cytogenetic and molecular heterogeneity in atypical chronic myeloid leukemia

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Chronic myeloid leukemia (CML) is characterized in 95% of the cases by the presence of the BCR/ABL fusion gene, that constitutively activates a tyrosine kinase, usually in association with the t(9;22) (q34;q11) translocation. However a small number of cases (5%) exist that appear to be quite similar to...
CML for clinical and haematological features but do not present the BCR/ABL chimeric protein; up to now this group of patients is included in the BCR/ABL negative CML or atypical CML or CML-like MPD group. We report 13 cases that were associated with a Philadelphia negative, BCR/ABL negative myeloproliferative disorder characterized by leukocytosis (consisting primarily of mature neutrophils and neutrophil precursor in the peripheral blood), presence of immature dysplastic myeloid cells in bone marrow and peripheral blood, low basophil count, monocytosis (<10%) and spleen enlargement. These features were consistent with a diagnosis of atypical chronic myelogenous leukemia. Cytogenetic, FISH and RT-PCR analysis were performed on bone marrow of all patients. Cytogenetic abnormalities were present in 6 out of 13 patients (46%). Trisomy 8 was observed in one case, a 22q- in another case, a t(8;22)(p11;q11) in two cases; a patient had a 12p- and a patient showed a t(6;11)(q15;q23) with a 20q- as additional anomaly arising in accelerated phase. The FISH analysis studies were performed to demonstrate the possible involvement of the BCR, ABL, FGFR1, PDGF-α, PDGF-β genes; to confirm the karyotype analysis; and to exclude the rearrangement of the MLL gene in the case with t(6;11). The t(8;22) was investigated using BAC 350N15, spanning the FGFR1 gene and BCR probes; both patients showed the BCR/FGFR1 rearrangement on chromosome 22 and one of the two patients also presented a deletion of the 3’ portion of BCR and 5’FGFR1 on chromosome 8. This result is the first demonstration that deletions on derivative chromosome 8 similar to those observed on derivative 9q+ in CML may occur also in the t(8;22) translocation. These data were further supported by the results of molecular biology analysis. RT-PCR analysis for BCR/ABL, ETV6/PDGF-β, FIPL1/PDGF-α, and BCR/FGFR1 transcripts was done on all patients and we found the BCR/FGFR1 transcript positive in the two patients with t(8;22). The other transcripts were negative for all patients. These results suggest that aCML is a rare disease with different and heterogeneous chromosomal abnormalities, in contrast with the typical CML that is distinguished by a specific marker, the Ph chromosome. This genetic heterogeneity has to be clarified in order to develop specific therapeutic strategies.

PO-133
CLINICAL SIGNIFICANCE OF NEUTROPHIL CD177 MRNA EXPRESSION IN PH-NEGATIVE CHRONIC MYELOPROLIFERATIVE DISORDERS
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The PRV-1 gene has been proposed as a molecular marker of polycythemia vera (PV). Recent studies have shown that PRV-1 and NB1 are alleles of the polymorphic gene CD177, which belongs to the Ly-6/uPAR superfamily. The coding regions of these alleles differ at only 4 nucleotides. We studied neutrophil CD177 mRNA levels in normal subjects and in 235 patients with Ph-negative chronic myeloproliferative disorders (CMDs), including PV, essential thrombocytopenia (ET) and myelofibrosis with myeloid metaplasia (MMM). Additional disease states were investigated for comparison. Highly variable neutrophil CD177 mRNA levels were observed in normal individuals. Neutrophils isolated from bone marrow or from peripheral blood following G-CSF administration showed higher CD177 expression than circulating granulocytes on steady state. Increased neutrophil CD177 mRNA levels were detected in all CMDs, and also in reactive conditions and in disorders such as chronic myeloid leukemia and myelodysplastic syndromes. In the differential diagnosis between polycythemia vera and secondary erythrocytosis, the assay sensitivity was only 20%. These findings indicate that an elevated neutrophil CD177 mRNA level is not a specific marker for the diagnosis of PV and neither of CMD. From a clinical viewpoint, neutrophil CD177 mRNA overexpression is rather a marker of abnormal neutrophil production and/or release.
High-dose chemotherapy with autologous peripheral blood stem cell transplantation (aPBSCT) has been used for the treatment of several malignancies because of a more rapid haemopoietic recovery than autologous bone marrow transplantation (ABMT), and also less supportive care and a shorter hospitalization. Myeloid growth factors (G-CSF and GM-CSF) were introduced into clinical practice to accelerate neutrophil recovery after PBSCT in order to shorten the duration of neutropenia, although there is no consensus about their indications and schedules of administration. Recently Peg-Filgrastim, a G-CSF form characterized by a increased plasma half-life, has been approved for clinical use. It was developed linking a 20-kd polyethylene glycol molecule to the N-terminal of the Filgrastim molecule. This modification implies a greater physical and thermal stability, the resistance to enzymatic degradation and the decreased renal clearance; therefore, neutrophil-mediated clearance becomes the predominant route of elimination; lower is neutrophil count, higher is drug plasmatic level. Recent clinical trial have shown that a single 6 mg dose of Peg-Filgrastim per chemotherapy cycle can reduce the duration of chemotherapy-induced neutropenia as safely and effectively as daily administered Filgrastim. Although in our institution the use of G-CSF after unmanipulated aPBSCT is not a routinely procedure, in order to verify the efficacy and feasibility of use of Peg-Filgrastim in transplant setting, we administered to patients submitted to aPBSCT for haematological malignancies a single subcutaneous dose of 6 mg Peg-Filgrastim on day +1 after stem cells infusion. In this study there were 8 patients, 3 female, 5 male, median age was 49 years (range 34–62), 5 affected by non-Hodgkin's Lymphoma, 3 affected by Multiple Myeloma; disease status at transplant was progressive disease in 2 patients, partial remission in 2 patients and complete remission in 4 patients. Conditioning regimens were BuMel (Busulfan 4 mg/kg on day -5 through -2; Melphalan 90 mg/m² on day -1) in 7 patients, HDMel (Melphalan 100 mg/m² on day -3 and -2) in 1 patients. On day 0 patients received a median of 7.4×10⁶/kg CD34+ (range 3.9-16.5×10⁶/kg). We evaluated haematological engraftment, and other clinical outcomes; all results are expressed as a median value in Table 1. All patients showed a prompt engraftment and were discharged on the average of 16 days after cell infusion. Peg-Filgrastim administration was well tolerated in all but 1 patient reporting bone pain for three days after drug administration. No erythema or other local lesion appeared at the site of the injection. Our preliminary data showed feasibility, safety and efficacy of Peg-Filgrastim administration after aPBSCT for haematological malignancies. Drug administration resulted in an expected acceleration of neutrophil recovery without detrimental effect on the overall engraftment providing, with single administration schedule, a better compliance and comfort for patient. Based on these data is now ongoing a larger study in order to assess the potential benefits of this approach on the other clinical outcomes such as sepsis occurrence, days of fever, antibiotic therapy, length of hospitalization and economical impact of Peg-filgrastim.

Table 1

<table>
<thead>
<tr>
<th>RESULTS</th>
<th>Median value</th>
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<tbody>
<tr>
<td>Days of PMN &lt; 0,1x10⁹/L</td>
<td>4 (3-5)</td>
</tr>
<tr>
<td>Days to PMN &gt; 0,5x10⁹/L</td>
<td>10 (7-11)</td>
</tr>
<tr>
<td>Days to PMN &gt; 1,0x10⁹/L</td>
<td>11,5 (7-15)</td>
</tr>
<tr>
<td>Days to Platelets &gt; 20x10⁹/L</td>
<td>11 (6-24)</td>
</tr>
<tr>
<td>Days to Platelets &gt; 50x10⁹/L</td>
<td>22 (12-38)</td>
</tr>
<tr>
<td>Days to Platelets &gt; 100x10⁹/L</td>
<td>26 (14-46)</td>
</tr>
<tr>
<td>Days to Haemoglobin &gt; 10 g/dL</td>
<td>30 (10-94)</td>
</tr>
<tr>
<td>Days to reticulocytes count &lt; 1%</td>
<td>16 (12-121)</td>
</tr>
<tr>
<td>Days of Hospitalization</td>
<td>23 (19-30)</td>
</tr>
<tr>
<td>No pRBCu</td>
<td>1 (0-2)</td>
</tr>
<tr>
<td>No SDu</td>
<td>1,25 (1-3)</td>
</tr>
<tr>
<td>Days body-temperature &gt; 38°C</td>
<td>4,5 (2-10)</td>
</tr>
<tr>
<td>Days of non-prophylactic antibiotic therapy</td>
<td>7 (6-12)</td>
</tr>
<tr>
<td>Bloodstream infection</td>
<td>3 patients</td>
</tr>
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</table>
T-cells play a key role in priming GVHD (graft-versus-host disease) or the immune response to allogeic transplanted organs. After encountering the antigen, T-cell fate can be apoptosis, anergy or activation. Currently the main immunosuppressive agents act inhibiting the various steps of T-cell activation pathway or causing lymphocyte depletion. ATG, antithymocyte globulin, held on the early success of solid organ transplantation in the 1960-70s, and in the 1980s, it found new roles combating GVHD in the bone marrow transplant unit and treating aplastic anemia. Recently, there have been reports that ATG also benefits some patients with myelodysplastic syndrome. Immunosuppressive therapies with combinations of ATG and chemotherapeutic drugs (cyclosporine, cyclophosphamide, fludarabine, ecc.) induce responses up to 70% of patients. However these responses can be often transient. An efficient way to improve antibodies effects is to conjugate them to toxic moieties, in order to obtain immunotoxins, chimeric proteins with specific efficacy. In this preliminary work we studied the cytotoxic effect of ATG conjugated to the ribosome-inactivating protein (RIP) saporin-S6. RIPS are plant toxins, whose enzymatic activity was identified as polynucleotide:adenosine glycosidase. Saporin-S6 and ATG were linked via a disulphide bond between chemically inserted sulphydryl groups and the conjugate was purified by gel filtration. After conjugation saporin-S6 maintained its enzymatic activity, evaluated on a cell-free protein synthesis system (rabbit reticulocytes lysate), and ATG was almost 10000 times less active. Unstimulated lymphocytes resulted much less sensitive to immunotoxin than activated cells (AC50 about 10 nM). ATG-saporin-S6 also resulted cytotoxic on several lymphoma cell lines. RIP incremented its toxicity on target cells by 3 logs upon conjugation with ATG. In all cell lines protein synthesis was completely inhibited by the immunotoxin at 10 nM concentration. This cytotoxicity was also confirmed by another extremely sensitive assay based on ATP detection.

**References**


**Posters**

**PO-135**

THE ATG-SAPORIN-S6 IMMUNOTOXIN CAUSE A COMPLETE DEPLETION OF ACTIVATED T CELLS

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Kaposi sarcoma (KS) is a vascular tumor of haemopoietic origin occurring in immunosuppressed patients, including AIDS and transplant patients. We have learned from studies of EBV-related PTLDs that the viral load should be combined with other diagnostic methods to increase its predictive value. Additional monitoring of the EBV-specific T-cell immune response may further improve the ability to predict PTLD in transplant recipients. The aim of the present study was to set up a specific and sensitive assay to evaluate the dynamics and prognostic value of monitoring HHV8-specific T cell immune response in addition to HHV8 DNA load in patients with KS. To assess the presence of HHV8-specific cytotoxic T-lymphocyte (CTL) activity, we adopted the single-cell IFN-gamma release EliSpot assay. Three previously identified HHV8-specific CBB HLA-A*02-restricted CTL epitopes (9-10 AA residues long), derived from one latent (K12) and two lytic (gH, gB) antigens, were used to stimulate PBMCs from an HIV-negative patient with KS receiving long-term immunosuppressive therapy including methotrexate, cyclosporin and steroids for rheumatoid arthritis. Both HHV8 CTL activity and viral load (real-time PCR) were evaluated over a six months period on sequential blood samples. HHV8-specific CD8 T cell response was detectable (89-100 SFC/10⁶ PBMCs) for all three peptides before KS, but became absent simultaneously the appearance of KS cutaneous lesions, indicating a correlation between a lower T cell response and the presence of disease. In contrast, HHV8 DNA load was poorly detectable (2-20 viral copies/ug DNA) over the time. In conclusion, in HIV-negative KS patients the predictive value of HHV8 load determination is limited. In these patients EliSpot assay may be considered a reliable method for monitoring HHV8-specific immune response providing a valuable parameter to guide therapy. We are currently assess-
ing the predictive value of EliSpot in transplant patients with KS.

PO-137
CYTOTOXIC ACTIVITIES OF HUMAN HLA IDENTICAL DONOR DERIVED CYTOKINE INDUCED CELLS (CIK) AGAINST ACUTE MYELOID BUT NOT LYMPHOID LEUKEMIA BLASTS
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Cytokine induced killer (CIK) cells have been described as strong lytic effectors against different types of solid tumors. Although CIK are CD3+ CD8+ CD56+ cells, they induce tumor lysis through a T cell receptor (TCR) independent recognition, through NKG2D receptor. Recently it has been reported that CIK cells are able to induce graft versus leukemia (GvL) but not graft versus host disease (GvHD). Aims of the study: to develop a reproducible clinical grade method to ex-vivo expand CIK cells from peripheral blood of HLA identical hematopoietic stem cell donors, and to characterize their lytic activity against acute myeloid and lymphoid blasts. Peripheral blood (30 ml) from HLA identical familiar (N= 9) or unrelated donors (N= 7) were collected at time of bone marrow donation or in case of G-CSF mobilized donors, at time of their clinical examination. After Ficoll separation, peripheral blood mononuclear cells (PBMC) were cultured in vitro in 10 mL of RPMI 1640, in the presence of 10% clinical grade FCS, 2nM Glutamine, Penicillin/Streptomycin and 1000U/ml IFN-γ (Gammakine®). The day after 50 ng/ml of anti-CD3 (OKT-3, Orthoclone) and 500 U/ml of IL-2 (Proleukin, ) were added to the culture and cells were maintained at a concentration of 0.5x10⁶ cells/mL in the presence of only IL-2. The cytotoxicity of CIK cells was determined by Calcein AM release assay. In our culture condition the proportion of CD3+ CD56+ cytotoxic cells, increased from 1-3% of total mononucleated cells of normal donors at day 0 up to 80% (median 55%) at day +21 of in-vitro culture. Moreover a median 10 times fold increase in the absolute cell count was observed, with a median 25 fold increase of CD3+ cells, while CD3+ CD56+ CD8- subset showed a 350 times fold increase. Starting from 30 ml peripheral blood a median number of 400 X 10⁶ CD3+CD56+ effector cells can be obtained over 21 days of in vitro culture. Cell sorting experiments using CD56 magnetic microbeads showed a strong cytotoxic potential against the standard NK target (K 562 cell line, median cytotoxicity at 4 hours of 45% at a E:T ratio of 10:1). Donor derived CIK cells were tested against HLA identical patient derived blasts (5 AML, 8 ALL). Cytotoxicity was documented only against myeloid targets, indipendently from their FAB classification (at 4 hours median cytotoxicity of 20% with a E:T ratio of 10:1). These experiments were performed under GMP conditions so to provide a protocol for further scaling up. Conclusion: HLA identical donor derived CIK cells can be always expanded ex vivo in a reproducible, clinical grade and effective way, showing a high cytotoxic potential against myeloid but not lymphoid leukemia blasts. These pre clinical data allow the design of new adoptive immunotherapy protocols for the treatment of acute myeloid leukemia patients relapsing after HLA identical allogeneic stem cell transplantation.

PO-138
MONITORING OF MYELOID AND PLASMACYTOID DC NUMBERS AND FUNCTION IN THE PERIPHERAL BLOOD AND BONE MARROW OF PATIENTS WITH CHRONIC GVHD FOLLOWING ALLOGENEIC HEMATOPOIETIC STEM CELL TRANSPLANTATION
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Although dendritic cells (DC) have been shown to initiate acute GVHD, their role in chronic GVHD is unknown. Human DC comprise at least two distinct subsets, i. e. myeloid DC, that have been associated with the triggering of alloimmune responses, and plasmacytoid DC, that have been shown to modulate alloimmune responses, through induction of Th2 or T regulatory activity. In this study we evaluated the number and function of mDC and pDC either circulating in the PB or residing in the BM of patients with or without chronic GVHD at > 100 days after HSCT. Patients with signs and symptoms compatible with chronic GVHD were included in the study, provided they had not received yet induction therapy with corticosteroids (n=18). Patients without chronic GVHD after > 100 days follow up were included as controls (n=8). Follow up was comparable among the two groups [median 303 days in GVHD patients (25th to 75th percentile 259-411) vs 393 (180-591) in controls (p=n. s. )]. Furthermore, no difference was observed among the two groups as regarding age, stem cell source, conditioning regimen, disease state and whether they were still taking non-steroidal immunosuppressive drugs. Peripheral blood samples from patients and controls were analysed for mDC (identified as BDCA1+ CD191-), pDC (identified as BDCA2+ and monocyte (identified as CD14+) numbers.
Lymphotactin and SDF stimulate T cells. Furthermore, serum levels of CXCR4 in GVHD patients were significantly lower in GVHD patients (0.5 (0.3-0.8) vs 1.5 (1.2-2.6) (p=0.03), suggesting selective loss of pDC.

PO-139 IMATINIB THERAPY IS THE FIRST THERAPEUTIC OPTION FOR CHRONIC MYELOID LEUKEMIA PATIENTS RELAPSED AFTER ALLOGENEIC STEM CELL TRANSPLANTATION


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We studied clinical and molecular follow-up of 16 patients with chronic myeloid leukemia (CML) relapsing after allogeneic stem cell transplantation (SCT). Disease at the time of treatment with Imatinib was in chronic phase (CP) in all but three patients who were in accelerated phase (2 patients) or in blastic crisis (one). All of them were in cytogenetic relapses. The median interval between relapse and start of Imatinib therapy was 34 months (7-156). Two patients had failed treatment with donor lymphocyte infusions prior to Imatinib; three patients had received therapy with IFNα and two with hydroxyurea (HU). All patients were treated with Imatinib (400 or 600 mg/daily), without significant hematological toxicity. The overall hematological response rate was 100% and the complete cytogenetic response (CCR) was 100% for all patients either relapsed in CP or AP. Complete molecular responses, intended as 3 logs reduction of BCR-ABL/B2M were obtained in 15/16 patients in three months, independent from the phase of the disease at the moment of relapse. One patient (UPN 1) achieved a CMR after 15 months of therapy. With a median follow-up after relapse of 19 months (range 4-32), the estimated 2-year survival for all patients was 100%. Mixed chimerism has been evaluated in 9 patients and after Imatinib therapy was assessed as full donor in all but one of them. We conclude that Imatinib has significant activity against CML in relapse after allogeneic bone marrow transplantation with durable cytogenetic remissions and significant molecular 3 log reduction of BCR-ABL/B2M obtainable in all patients. Imatinib therapy is the first therapeutic option to be proposed for chronic myeloid leukemia patients who relapse after allogeneic stem cell transplantation.

Funding: FIRB 2001; COFIN 2003, Fondazione del Monte di Bologna e Ravenna, AIL, AIRC, Ateneo di Bologna 60% (Baccarani).
In haploidentical transplants, T cell depletion is essential for preventing GvHD but it is responsible for slow T cell recovery and 35% infection-related mortality. Therefore, new strategies to protect patients from infections, while not causing GvHD, are necessary. Conditioning regimens which include alloreactive NK cells could be a new strategy for improving immune reconstitution. In murine MHC mismatched BMT models they allowed for the concomitant transfer of high doses of T cells without causing GVHD. Aim of the study: assessment of immunity to pathogens in mice given standard T cell-depleted mismatched BMT vs mice given alloreactive NK-based conditioning and T cell-replete mismatched BMT. After transplant, a low virulence strain of Candida Albicans was infused to determine whether 1) mice survived infection and 2) specific T cell immunity could be elicited (vaccination) to a challenge with a high virulence Candida Albicans strain given two weeks after vaccination. The challenge would be fatal in healthy non-vaccinated mice. Mice receiving standard conditioning (TBI) and T cell-depleted mismatched BMT vs mice given alloreactive NK-based conditioning and T cell-replete mismatched BMT. After transplant, a low virulence strain of Candida Albicans was infused to determine whether 1) mice survived infection and 2) specific T cell immunity could be elicited (vaccination) to a challenge with a high virulence Candida Albicans strain given two weeks after vaccination. The challenge would be fatal in healthy non-vaccinated mice. Mice receiving standard conditioning (TBI) and T cell-depleted mismatched BMT vs mice given alloreactive NK-based conditioning and T cell-replete mismatched BMT. After transplant, a low virulence strain of Candida Albicans was infused to determine whether 1) mice survived infection and 2) specific T cell immunity could be elicited (vaccination) to a challenge with a high virulence Candida Albicans strain given two weeks after vaccination. The challenge would be fatal in healthy non-vaccinated mice.
predominant TCR peak preceded or appeared concomitantly to the achievement of the IgH-negativity. On the other hand, 4 cases converted back to the IgH-negativity during follow-up; interestingly, in two of them the area of a clone previously characterized as recipient-specific resulted significantly increased. These results suggest that different T populations sustain GVM and GVHD and that analysis of TCR spectratype could be a useful tool for monitoring patients who underwent non-myeloablative allogeneic transplant.

**PO-142**

**REDUCED INTENSITY CONDITIONING REGIMEN FOR ALLOGRAFTING FOLLOWING CYTOREDUCTIVE AUTOGRAFTING FOR METASTATIC BREAST CANCER**

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**Background:** The development of reduced intensity conditioning regimens (RIC) has decreased the risks of allografting to a level warranting such investigational trial. For patients without sibling donors, autografting has been explored as a method to enhance the efficacy of chemotherapy in advanced solid tumors. This pilot study combined both the procedures in order to maintain the benefit of both approaches with acceptable toxicity. **Methods:** The feasibility of this tandem transplant procedure was evaluated in 17 patients with metastatic breast cancer. At a median of 51 days after autografting, the patients received a RIC followed by donor mobilized HLA identical peripheral blood progenitor cells. Donor lymphocyte infusions were given to 12 patients with stable mixed chimerism and/or progressive disease who did not show signs of aGVHD. **Results:** Eleven (64%) out of 17 patients achieved sustained engraftment. Three patients who were resistant to conventional therapy achieved PR after autografting and CR after allografting; another no-responsive patient achieved PR for an overall response rate of 4/17 (22%). No early TRM was observed during the first days. Grade II acute GVHD occurred in 5 patients (29%) and extensive chronic GVHD in 5 patients (29%). All the responses were accompanied by the occurrence of GVHD. At April 2004, 5 out of 17 patients (29%) are alive 90-2160 days (median, 1320 days) from this 2-step approach. **Conclusions:** Tandem transplant is a feasible procedure in metastatic breast cancer patients; and the exploitation of GVHD is a promising finding.

**PO-143**

**DIFFERENCES BETWEEN QUANTITATIVE CMV-PCR IN PLASMA AND CMV ANTIGENEMIA ASSAY IN MONITORING PATIENTS AFTER ALLOGENEIC STEM CELL TRANSPLANTATION (SCT)**


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Human cytomegalovirus infection (HCMV) represents the most common and potentially severe viral complication in patients undergoing hematopoietic SCT; therefore, the effective anti-CMV treatment strategy must be based on a sensitive and reliable diagnostic assay to rapidly evaluate active CMV infection. Pp65 antigenemia (pp65Ag) is considered the standard assay to detect CMV infection; however, whether PCR assay for detection of CMV DNA in plasma may anticipate pp65Ag is still controversial. The aim of the study was to investigate the usefulness of a quantitative plasma PCR test and compare it with the pp65Ag for detection of CMV infection following SCT. Between December 2002 and June 2004, 28 patients underwent an allogeneic SCT from either an HLA-identical sibling (23) or an unrelated donor (5). All patients were weekly monitored for CMV infection by both quantitative CMV-PCR in plasma (COBAS AMPLICOR CMV MONITOR Test with lower limit detection of 600 copies/ml plasma) and pp65 Ag during the first 100 days after SCT. As CMV prophylaxis, Acyclovir was given either at standard or high dose in related or unrelated transplants, respectively. No patients received specific Ig, Gancyclovir, Foscarnet or Cidofovir as CMV prophylaxis. Pre-emptive therapy with Gancyclovir or Foscarnet was started at the first detection of antigenemia (> 1 positive cell). Plasma CMV DNA was not considered for clinical decision making. Overall, 20/28 patients (71.4%) had CMV infection within 100 days from SCT: in 11 patients, CMV was detected by pp65 alone, in 8 by both methods and in 1 by PCR alone. Pp65Ag positivity after SCT was earlier (median 41 days, range 0-100) than plasma PCR assay (median 48 days, range 5-55). A total of 369 blood samples were analyzed. CMV was detected in 45 samples (12%) by a single or both methods. PCR detected a median of 1230 copies/ml (range 770-22600) while pp65Ag showed a median of 6 positive/150000 total PMN examined (range 1-400). Overall, 15 samples were found to be positive by PCR and pp65-antigenemia, 25 samples were pp65Ag positive but PCR CMV negative, 5 was PCR positive/pp65Ag negative, and 324 were negative by both assays. Three patients developed CMV disease (10.7%) detected by both methods in 2/3 cas-
es; only 1 patient developed intestinal CMV disease responding to antiviral therapy, despite negative pp65Ag and PCR assays. Three patients died of transplant related mortality (1 infection, 1 grade IV aGVHD, 1 CMV disease + aGVHD) and 3 of disease progression. In conclusion, pp65Ag detected CMV infection earlier than plasma PCR in our allogeneic SCT recipients.

PO-144
PHASE II STUDY OF A SINGLE PEGFILGRASTIM INJECTION AS ADJUNCT TO CHEMOTHERAPY TO MOBILIZE STEM CELLS INTO PERIPHERAL BLOOD OF RESISTANT/RELAPSED LYMPHOMA PATIENTS

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The primary end point of the study was the successful mobilization of a target cell dose of $2 \times 10^6$ CD34$^+$ cells/kg in lymphoma patients receiving ifosfamide, epirubicin and etoposide (IEV) chemotherapy and a fixed dose (6 mg) of pegfilgrastim given as single subcutaneous injection. An open-label phase II study including 25 relapsed or refractory patients (Hodgkin’s disease=4; aggressive non-Hodgkin’s lymphoma=21) was conducted to evaluate the efficacy of pegfilgrastim, in combination with salvage chemotherapy, mobilizing CD34$^+$ stem cells into peripheral blood. Following chemotherapy, all patients had grade 4 neutropenia with a median duration of 1.5 days (1-3). Pegfilgrastim treatment was well tolerated and only 2/25 patients required pain-control medication. CD34$^+$ cells were mobilized in all patients. The median (range) peak value of peripheral blood CD34$^+$ cells after IEV chemotherapy and pegfilgrastim was 141/µL (12.8-386) and occurred almost invariably on day +14 (13-16). Twenty three/25 patients underwent a single apheresis to collect a median of 8.7 CD34$^+$ cells/Kg (1.8-17.3). Twenty four/25 patients (96%) reached the target cell dose of $2 \times 10^6$ CD34$^+$ cells/Kg. High concentrations of circulating CD34$^+$ cells ($> 50/µL$) were observed for several days after the achievement of the peak value. Sixteen patients have been transplanted so far with pegfilgrastim-mobilized CD34$^+$ cells and all of them showed a rapid and sustained engraftment after high-dose chemotherapy. Our results show that pegfilgrastim as adjunct to chemotherapy is a predictable and highly effective mobilization regimen in lymphoma patients.

PO-145
TRANSFERRING FUNCTIONAL IMMUNE RESPONSES TO PATHOGENS ACROSS THE HLA BARRIER AFTER MISMATCHED HEMATOPOIETIC TRANSPLANTATION

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Re-building immunity to pathogens after full HLA haplotype-mismatched transplant could be hastened by transferring immune T lymphocytes. The obstacle is lethal GVHD. We generated over 1,200 Aspergillus-specific CD4$^+$ clones from 10 donors and over 3,000 CMV-specific CD4$^+$ clones from 25 CMV-positive donors. 1–10% of clones displayed cross-reactivity to recipient alloantigens. Non recipient-reactive clones were used for adoptive therapy. Transplant recipients were given a single shot of adoptive therapy (10 with Aspergillus-specific cells and 25 with CMV-specific cells) ranging from $10^5$ to $3 \times 10^6$/Kg cells on day +20 after transplant. The only case of GvHD occurred in the patient who received $3 \times 10^6$/Kg cells. In a control group of 46 recipients who had not received adoptive therapy, Aspergillus- and CMV-specific T cells were first detected 9–12 months after transplant (by LDA). In the control group of 13 recipients, who had not received Aspergillus adoptive therapy, all displayed positive Aspergillus Galactomannan antigenemia which persisted for several weeks after transplant. Six of 13 had pneumonia and all of them died of aspergillosis. In the 10 transplant recipients who received adoptive therapy with anti-Aspergillus clones all displayed positive antigenemia. After adoptive therapy antigenemia decreased significantly in all of them. All of the 10 patients had aspergillosis and only 1 died. The remaining 9 cleared the disease. In the CMV adoptive therapy study, 30/33 patients who did not receive anti-CMV T-cell clones underwent repeated CMV reactivation. 12/33 developed CMV disease, lethal in 9. In the 25 patients who received CMV-specific cells, there was a quick (2–3 wks) recovery of CMV-specific specific T cells that remained stable over time (monitored for ~1 year). CMV antigenemia became negative from 2–3 weeks after the infusion onwards in 18 patients. Seven patients had CMV disease at the time of the infusion. Five cleared the disease and 2 died. None of the other 18 patients developed CMV disease. Finally, in 2 transplants from CMV-negative donors into CMV-positive recipients, we used pre-transplant recipient cells to obtain CMV-specific, non donor-reactive clones. Their post-transplant infusion did not cause
rejection or cytopenia, was followed by prompt and stable recovery of CMV-specific T cells, and controlled CMV reactivation. DNA polymorphism indicated CMV-specific clones were of recipient origin, unlike the other T cells which were of donor origin. This study identified safe and effective ways to re-build immunity to pathogens after haploidentical transplantation.

**PO-146**

**IMMUNOREGULATION MEDIATED BY BONE MARROW-DERIVED MESENCHYMAL STEM CELLS (BM-MSC). EVIDENCE THAT BM-MSC CAN EXHIBIT BOTH IMMUNOSUPPRESSIVE AND IMMUNOSTIMULATORY EFFECT**

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It has been reported that bone marrow-derived mesenchymal stem cells (BM-MSC) exert a strong immunosuppressive effect on mixed lymphocyte reaction (MLR). This effect is thought to be mediated by immunosuppressive cytokines including transforming growth factor (TGF)-β and/or hepatocyte growth factor (HGF). This immunosuppressive effect may favour both engraftment of bone marrow transplantation and decrease the incidence of graft versus host reaction. To better understand the functional consequences of BM-MSC/lymphocytes interaction, we analysed whether BM-MSC can activate, rather than inhibit, lymphocyte subsets. To this aim we obtained BM-MSC from either healthy donors (n=4) or from patients (n=10) suffering from acute myeloid leukemia (AML) in post-chemotherapy complete remission. BM-MSC did not express typical hematopoietic markers such as CD34, CD45 and CD14 but bore CD105, CD73, CD29, CD44, CD54, HLA-A B C antigens. In addition, they did not express CD133. We found that BM-MSC, used as stimulators, trigger the production of TNF-α, but not of IFN-gamma, by allogeneic peripheral blood mononuclear cells (PBMC) upon 24 hrs stimulation. Importantly, this effect was evident in 3 out of 7 BM-MSC/PBMC co-cultures. In these PBMC, activation markers including CD25 and CD69 were highly upregulated. Indeed, BM-MSC can function as good stimulator in co-culture with allogeneic PBMC. Experiments aimed to define whether this phenomenon is linked to the different HLA class I antigens expressed by BM-MSC and PBMC are in progress. BM-MSC populations constitutively produced TGF-β and strongly inhibited (up to 90%), in a dose dependent manner, MLR when used as a third party. Interestingly, we noticed a strong expansion of NK and T lymphocytes expressing CD94 in MLR in the presence of BM-MSC suggesting that these cells can favour the proliferation of lymphocytes bearing members of Inhibitory Receptor Superfamily (IRS), resembling the reported expansion of NK and/or T cells observed in patients early after bone-marrow transplantation. Altogether, these findings indicate that BM-MSC can also activate, beside inhibit, different lymphocytes subsets. Thus, a deeper analysis of the molecular mechanism underlying their interaction with lymphocytes is mandatory, prior to employ them in therapeutic trials.

**PO-147**

**INCIDENCE, OUTCOME AND RISK FACTORS OF CHRONIC GVHD AFTER UNRELATED DONOR STEM CELL TRANSPLANTATION: A SINGLE CENTER EXPERIENCE**


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Chronic graft-versus-host disease (GVHD) is an important cause of late morbidity and mortality after allogeneic stem cell transplantation (SCT). Its incidence and severity are increased by the use of matched unrelated donors (MUD) and of peripheral blood as stem cells source, which is becoming more and more frequent. The purpose of this retrospective study was to analyze incidence, outcome and risk factors of chronic GVHD after MUD-SCT. 46/57 patients who underwent SCT from unrelated donor were evaluable for chronic GVHD. Median age was 31,5 (14–56) years and median follow-up was 18 (4–77) months. CyA was administered intravenously (i. v.) in continuous infusion from the day before transplant (3 mg/kg/day) and orally at i. v. equivalent dose from 4th week after SCT. MTX was administered i. v. on day +1 (15 mg/mq) and on day 3, 6, 11 (10 mg/mq). CyA was tapered from day 100 or according to clinical assessment. In all patients CyA was monitored for steady-state levels during the period of continuous infusion and for through levels during the period of oral intake. Several independent variables were analyzed as possible risk factors for the development and the severity of chronic GVHD: sex, age and status of disease at SCT, source of stem cells, infused cell dose, sex mismatch, CMV reactivation, acute GVHD. The hazard ratio for other variables (1.mean CyA blood concentration during 1st, 2nd, 3rd, 4th and 5th month and 2. rate of CyA concentration during 2nd, 3rd, 4th
High Serum Leptin in Patients with Chronic Graft versus Host Disease After Hematopoietic Stem Cell Transplantation

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Leptin is an adipocyte-derived hormone of the long-chain helical cytokine family playing a regulatory role in the neuronal control of body weight by inhibiting food intake and stimulating energy expenditure. Recently, it has been documented that leptin exerts a direct effect on T-lymphocyte responses, promoting Th1 and suppressing Th2 cytokine production. Increased serum leptin values have been found after heart, liver, kidney and stem cell transplantation (SCT). The reasons and the mechanisms responsible for such a post-transplant increase are still unclear. We measured serum leptin concentration in a group of 60 transplanted patients and in 60 healthy subjects with similar age and body mass index (BMI). Relationships with age, gender, body mass index (BMI), gonadal status, lymphocyte subpopulations, Th1 and Th2 cytokine secretion, inflammation and time elapsed since transplant were investigated. Serum leptin level was significantly higher in transplanted patients compared to controls; in addition, it was higher after allo-SCT than after autologous (auto)-SCT, expressed as absolute value and after normalization for BMI. In 15 patients who were prospectively evaluated prior to and after allo-SCT, pre-transplant levels overlapped those of controls, while after SCT serum leptin increased two- to ten-fold. The typical sexual dimorphism (higher leptin in females) persisted in both transplant settings but was set up at higher levels. Significantly higher leptin values were found in 21 patients affected by chronic graft versus host disease (cGVHD) compared with those free of this complication. The physiological correlation with BMI was lost in the allogeneic setting, indicating a strong influence of factors other than the nutritional status on circulating leptin. No relationship was found between serum leptin levels and time from transplant, age, cortisol, C-reactive protein, and T-lymphocyte CD4-to-CD8 ratio. Among the cytokines secreted by lymphocytes (IL-4, IL-5, IL-10, IL-2, IFN-γ and TNF-α), only serum IFN-γ was found higher in allo-SCT patients compared to auto-SCT patients and normal controls. Within allo-SCT patients, mean IFN-γ levels were higher in patients with than in patients without cGVHD. Moreover, a significant correlation between leptin and IFN-γ was found only when allografted patients with IFN-γ values >15 pg/mL were considered. Based on the evidence that leptin increase may contribute to the onset of immune response, we also investigated in vitro consequences of leptin blockade on T cell activation in a mixed lymphocyte reaction (MLR) from 5 HLA-mismatched couples of responder and stimulator T-lym-
Phocytes. Anti-leptin blocking antibodies partially inhibited T cell activation in MLR, suggesting a link between leptin and T-lymphocyte activation in the allo-SCT setting. In conclusion, we found greater increase in serum leptin after allo-SCT than autologous SCT, with the highest levels in patients with cGVHD. Loss of the physiological relationships between leptin, BMI and gonadal function, the correlation between serum leptin and IFN-γ levels in the allo-genetic setting, and the marked inhibitory effect of leptin-blockade on T cell activation in primary MLR suggest that T-cell activation after allo-SCT may be responsible for the leptin increase, which in turn might subsequently contribute to the maintenance of the immune attack leading to cGVHD.

**PO-149 SUCCESSFUL OF COLLECTION PERIPHERAL STEM CELL WITH PEG-FILGRASTIM IN RELAPSED LYMPHOMAS**


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Pegfilgrastim comprises the protein filgrastim to which a 20 kD polyethylene glycol (PEG) molecule is bound covalently to the N-terminal methionine residue of filgrastim. The addition of the PEG molecule reduces renal clearance of pegfilgrastim (PF) and increases the serum half-life more than filgrastim. This longer half-life facilitated neutrophil recovery with fewer injections. Several studies show that a single injection of PF is effective to induce neutrophil recovery following severe neutropenia after chemotherapy. Few data are now available on the efficacy of PF in the recovery of granulopoiesis in patients undergoing ABMT or marrow donors for BMT. In an ongoing study we present our experience on a series of patients with relapsed lymphomas planned for PBSCT. With the aim to present the feasibility and the tolerability of PF we analyse our preliminary data since January 2004 up to May 2004. The selection of patients was focused on those already treated by almost one line of therapy and relapsed or progressed; the salvage treatment plan included the PBSCT as part of sequential therapy with a debulky phase and consolidation with high dose chemotherapy rescued by PBSC. The study consisted on the administration of PF one single dose of 6 mg 5 days following DHAP scheme which consisted of Desametasone 40 mg/day over four days, Cisplatin 100 mg/m² over 24 hours continuous infusion and Cytarabine 2000 mg/m² b. d. The evaluation of CD34 cells was done daily starting from the day after the administration of PF up to the 3 day of collection of a minimum cumulative number of CD34 cells of 2.5×10⁶/Kg. Data are available on 7 patients, 4 males and 3 females, mean age 54 years (range 32 to 72 years). The diagnosis consisted of 3 non-Hodgkin’s lymphomas of B origin with an high-grade histology, 2 B non-Hodgkin’s low-grade lymphomas, 1 Hodgkin’s disease and 1 Breast metastatic cancer. All patients were previously treated by a combination chemotherapy showing some response but all were with active disease at the time of study started. The results show that all patients reached an adequate number (> of 20/µL) of circulating CD34 cells with a range from 9 to 11 days from the end of chemotherapy DHAP and 4 to 6 days from the administration of PF. All patients needed only one procedure of apheresis and the collected number of CD34 cells was 8.4×10⁶/Kg (range 4.5 to 26.3), with a median of CD34 cells of 70/µL (range 54 to 192). One patient was already successfully infused with PBSC, while the other patients haven’t yet undergone PBSC transplantation. Adverse events related to PF were mild to moderate bone pain. Our conclusion is that PF is safe and effective for collection of CD34 cells finalized to PBSCT. The preliminary data suggest that the peripheral blood progenitor cell mobilization by PF are comparable or greater than those achieved with daily filgrastim and the convenience of the single dose of PF may improve patients’ complianc and quality of life.

**PO-150 NEW NON-VIRAL METHOD FOR GENE TRANSFER INTO NORMAL AND TUMOR BONE MARROW-DERIVED PRIMARY CELLS**


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The availability of genetically modified cells is an essential prerequisite for many scientific and therapeutic applications including functional genomics, drug development, and gene therapy. Unfortunately, the efficient gene transfer into primary cells is still problematic. Whereas viral strategies are time consuming and involve safety risks, non-viral methods proved to be inefficient for most primary cell types. The Nucleofector technology is a novel gene transfer technique designed for primary cells and hard-to-transfect cell lines. In this study we tested the potential of the Nucleofector technology on different normal and tumor human BM-derived primary cells like CD34+ cells, mesenchymal stem cells (MSC), dendritic cells (DC) and leukemic-derived DC. By means of pEGFP expression vector (circular and linearized) we show high transfection efficiency within all the cell populations analysed ranging from 70% in CD34+ to 24% in DC. Cell toxicity was variable mainly depend-
Phenotypic analysis revealed no substantial antigenic alterations at different timepoints after nucleofection in all cells tested at the functional level. Nucleofected MSC still retained the potential to differentiate in both adipocytes and osteocytes while AML-DC could still induce an allogenic T cell response even if at a lower extent compared to non-transfected cells. Moreover, the nucleofection of AML-DC with a IL-12 encoding vector produced significant amount of the biological active cytokine as evaluated by ELISA. In conclusion, the Nucleofactor technology appears to be a useful tool for the engineering of hematopoietic and non-hematopoietic BM-derived cells.

Poster
STEM CELL TRANSPLANTATION II

PO-151
COMPLETE CLINICAL REMISSION AFTER HIGH-DOSE IMMUNE SUPPRESSION AND AUTOLOGOUS HEMATOPOIETIC STEM CELL TRANSPLANTATION (HSCT) IN SEVERE REFRACTORY CROHN'S DISEASE

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Crohn’s disease (CD) is an immune-mediated disease but it is not clear that autoimmunity is the underlying pathogenesis. Steroid is the main treatment but 30% of patients are dependant or resistant to this drug. In the last years antibody to TNF have been introduced in the treatment of acute steroid resistant disease and for maintenance of disease; despite good response 20 to 30% of patients are or become refractory. Recently high-dose immunosuppressive therapy with HSCT has shown to determine long clinical. We report a case of a colonic Crohn’s Disease (CD) refractory to immunosuppressive therapy in a 53- yrs-old man who underwent autologous HSCT with selected CD34+ cells. CD was diagnosed on February 2001 because of bloody diarrhoea, severe oral and pharingeal aphtous lesions and pyoderma. The patient had first received a course of methylprednisolone obtaining a clinical remission, but when treatment was stopped relapse was observed. Steroids together with azathioprine were then started but the appearance of severe arthritic pains and myalgias lead to the suspension of azathioprine. Infliximab was started at dosage of 5 mg /kg at 0, 15 and 45 days with a complete clinical response (CDAI < 150). A maintenance treatment with Infliximab was continued every 8 weeks, but after the first year of treatment the duration of the response gradually shortened, so on March 2003 methotrexate (25 mg i. m weekly) was added to Infliximab. On April 2003 the patient had a severe clinical and colonscopic relapse with extension of the disease to the rectum. The steroid dependence, the refractoriness to MTX, the intolerance to azathioprine associated with the shortening of time response duration to Infliximab were considered an indication to surgery (temporary colostomy or left colectomy with definitive colostomy).
my), which the patient refused. HSCT was then proposed. On July hematopoietic peripheral stem cells were mobilized using cyclophosphamide 2 g/m² and G-CSF (lenograstim) 10 mcg/kg. A total of 9.9×10⁹/kg CD34+ cells were collected by two aphereses procedures and the cells were then enriched by CD34+ positive selection using the CliniMAX device. On August the patient underwent high-dose immunosuppression with cyclophosphamide 50 mg/kg on days -5,-4,-3 and -2 and equine ATG (Merieux ®) at the dosage of 90mg/kg from day -4 to day -2 followed on day 0 by the infusion of 3.3×10⁹/kg CD34+ selected cells. The post transplant course was uneventful with only one episode of culture-positive fever (staph. Epidermidis) treated with specific antibiotic therapy while he was neutropenic. Severe neutropenia (ANC<500/L) lasted ten days while platelets never went under 25×10⁹/L. The transfusional support consisted of 2 irradiated packed red cells. Following HSCT, the patient remained on clinical remission. On October a new colonoscopic evaluation showed a reduction of the degree and the extension of the lesions and healing of mucosa in the rectum. Histologically a clear improvement was also observed. After one year from HSCT obtained: 1) a complete clinical response with any of the previous treatments. In this case it is important to underline that the improvement obtained in the rectal lesions could allow, in future, a less invasive surgical intervention (colo-recto anastomosis). This case confirms the possible role of HSCT in the management of severe steroid-resistant CD. The presence of extraintestinal symptoms which suggest a more relevant autoimmune component could be one of the criteria for selecting patients with severe resistant CD disease for HSCT.

We report clinical outcome and results of chimerism analysis in 64 patients (37 male and 27 female) with different malignancies (9 HD, 17 NHL, 16 MM, 3 CML, 4 AML, 1 CLL, 1 PTI, 7 Kidney and 6 Breast cancer) that received different non myeloablative conditioning, followed by allogeneic SCT: 22 Flu/Mel, 19 Flu/TBI and 23 Flu/Cy. The median age was 47 years (range 22–60). All but three received unmanipulated PBSC grafts from HLA-identical siblings donors mobilized with G-CSF; median number of infused CD34+ cells was 3.30×10⁶/kg (range 0.90-10.61). GVHD prophylaxis consisted of CSP/MTX or CSP/MMF (mainly used in patients with MM). Patients were monitored for chimerism using multiplex PCR amplification of fifteen STR loci and the Amelogenin locus. Donor engraftment was evaluated on unfractionated peripheral blood (PB), bone marrow samples and on immuno-magnetically selected PB CD3+ cells, at day +15, +30, +90 and so on. Median follow-up was 18 months (1-36). Kinetics of engrafting donor cells was different in the three conditioning regimens. The rate of complete donor chimerism (CDC=>95% donor cells) in FLU/MEL, FLU/TBI and FLU/CY at days +30 were 76%, 22%, 0%, respectively; at days +90 were 95%, 60%, 0%. In Flu/Mel protocol for HD patients (pts) the rate of CDC at days +30 was 100%; for NHL pts at days +80, +90 were 80%, 100% respectively. In Flu/TBI protocol for MM pts at days +30, +90 +180 were 43%, 64%, 100%. In Flu/Cy protocol for NHL pts at days +30, +90 +180 were 25%, 25%, 100%; for Kidney pts at days +30, +90 +180 were 0%, 14%, 29%; for Breast pts at days +30, +90 +180 were 0%, 0%, 0%. Acute GVHD occurred in 5/22 (23%) pts undergoing Flu/Mel, in three of them were grade > II (2 died), while 7/22 (32%) developed chronic GVHD, none of them died of it. In four (21%) pts treated with Flu/TBI protocol, grade III–IV acute GVHD was observed (2 died), chronic GVHD occurred in 9/19 (47%) pts and one of them died of it. Finally in Flu/Cy protocol acute GVHD was absent, while 4/23 (17%) pts developed chronic GVHD. Donor lymphocyte infusion (DLI) were given to 10 pts who had progressive disease (2 developed GVHD and one of them Complete Remission), and to 7 pts for mixed chimerism (none of them developed CDC). Thirty patients (47%) are alive to date. Our study shows that Flu/Mel protocol has unincreased transplant related mortality, mainly due to higher incidence of acute GVHD, while chronic GVHD occurred in all three protocols. We cannot evidenciate acute GVHD in patients that underwent Flu/Cy conditioning regimen because at day +90 none of them achieved complete donor chimerism.

PO-152
KINETICS OF CHIMERISM, GRAFT-VERSUS-HOST DISEASE AND DONOR LYMPHOCYTE INFUSION IN DIFFERENT NONMYELOABLATIVE PREPARATIVE CONDITIONING REGIMENS
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154 Posters
PO-153
A CASE OF REFRACTORY MYCOSIS FUNGOIDES RESPONDING TO ALLOGENEIC TRANSPLANT IMMUNE EFFECT
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A 36 year-old man with chronic dermatitis presented in October 2001 with a 2 cm diameter erythematous plaque on the left side of the neck. Routine laboratory tests, including serum lactate dehydrogenase (LDH) were within normal range. A biopsy was performed and a diagnosis of large-T-cell mycosis fungoides was made. A comprehensive work-up, including a trephine marrow biopsy and a total-body CT scan did not reveal other disease localizations. The patient received 12 courses of MACOP-B (methotrexate, adriamicin, cyclophosphamide, vincristine, methyl-prednisolone and bleomycin) followed by involved-field radiotherapy (30 Gy) attaining a 2 months regression of the neck skin lesion. In May 2001, the patient developed a new nodular plaque on the upper external side of the right leg. A new biopsy confirmed the disease progression and a secondline chemotherapy with DHAP (dexamethasone, cytarabine and cis-platinum) was started. However new skin lesions soon developed, and the patient was shifted to the IGEV combination (ifosphamide, gemcitabine, epirubicin and etoposide), with a partial regression of the skin lesions. Following the third IGEV course, as soon as the CD34+ cells rapidly peaked in the peripheral blood during the G-CSF priming, a stem cell apheresis was performed (collected CD34+ cells = 9.4 x 10^6/kg). In December 2002, after high-dose (180 mg/sqm) melphalan the patient was rescued with his peripheral blood stem cells. Engraftment was rapid and complete, but no disease response was obtained. The patient had an HLA-identical sister, who was primed with G-CSF 10 mg/kg/day subcutaneously, and her peripheral blood stem cells collected by apheresis on day 5.In February 2003, the patients was conditioned with anti-CD52 MoAb (Campath-1H) 30 mg/day for 3 days, followed by thiothepa 10 mg/kg (day -6), cyclophosphamide 30 mg/kg (dd -4, -3) and fludarabine 30 mg/m² (dd -4, -3). As graft he received 8.9 x 10^6/kg CD34+ cells from his sister peripheral blood. GVHD prophylaxis consisted of CSA 2 mg/kg IV from day -1 to +12, followed by CSA 4 mg/kg orally from day 12. PMN (>0.5 x 10^9/L) and platelet (>20 x 10^9/L) engraftment occurred on day 10 and 11, respectively. Skin lesions however, remained unmodified during the following 3 months, and CSA was therefore tapered and withdrawn on day +42. Since the skin lesions still persisted unmodified, and there was no evidence of GVHD, the patient received a treatment course with his donor lymphocyte infusions. One donor lymphocytes infusion (DLI) was performed on day +84. Extensive chronic cutaneous and mucous GVHD was observed 2 months after lymphocyte infusion (+140). Shortly after, a complete regression of the hematologic picture, confirmed by skin biopsy was attained. GVHD was effectively controlled by metil-prednisolone (0.2 mg/kg) tapered and than withdrawn 3 months after. Late infective complications consisted in 3 episodes of bacterial pneumonia occurred in the last 2 months. He has been followed as outpatient and at the present time he is disease free, at 15 months after allogeneic BMT. The course of MF is generally indolent, except when transformation to a large T-cell lymphoma occurs (>25% large cells on biopsy). Age (>60 y) and extracutaneous spreading were found to be associated with poor prognosis, while mean survival from transformation to death is 19.4 - 22 months in the reported series (Diamandidou E, 1998, Blood; Vergier B, 2000, Blood). In our case, graft-vs-tumor effect controlled the disease and induced a long-term remission. Whether this effect was potentiated by Campath-1H is difficult to establish. We suggest a potential role of Campath-based nonmyeloablative SCT in patients with large-cell transformed mycosis fungoides.

PO-154
HIGH DOSE-THERAPY WITH AUTOLOGOUS PERIPHERAL BLOOD STEM CELL TRANSPANTATION FOR WALDENSTROM’S MACROGLOBULINEMIA
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Waldenström’s macroglobulinemia (WM) is an incurable rare B-cell malignancy. Standard doses of alkylating agents or purine analogues effect response rates of up to 50%; however, CR are infrequent and there are no cures. Recently, anti-CD20 monoclonal antibody therapy has been successfully used in the treatment of WM, while the role of High-Dose Therapy (HDT) followed by Stem Cell Transplantation (SCT) in WM has not been established. AIM: to evaluate the efficacy and feasibility of an up-front strategy of HDT, serotherapy and Autologous Peripheral Blood Stem Cell Transplantation (APBSCT) for WM. MATERIAL AND METHODS: Between April 2001 and October 2003 six male patients (pts) with symptomatic WM were enrolled in an open-label trial of HDT (CHOP 3 courses, Rituximab 375 mg/m² x 4, CTX 4
PO-155
IMATINIB MESYLATE (GLIVEC) PRE-TREATMENT DOES NOT INFLUENCE THE OUTCOME OF ALLOGENIC HEMATOPOIETIC STEM CELL TRANSPLANTATION IN PHILADELPHIA-POSITIVE LEUKEMIAS

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As the role of Imatinib mesylate (Glivec) is gaining ground in the treatment of Philadelphia-positive (Ph+) leukemias, its effects on subsequent allogenic stem cell transplantation (SCT) are still largely unknown. Some recent reports seems to indicate a higher transplant-related mortality in patients pre-treated with Imatinib for Ph+ acute leukemias, but other studies on Ph+ acute lymphoblastic leukemia (ALL) patients do not confirm this data. We report our experience with Imatinib therapy preceding allogenic SCT in patients with Ph+ leukemias (ALL or CML). Ten patients (4 ALL and 6 CML) were treated with Imatinib before allogenic SCT at our Institution. The median age at diagnosis was 40 (range: 30-55) years. The ALL patients received 2 to 4 courses of chemotherapy; Imatinib therapy was started when hematologic recovery after the fourth cycle was documented, or after the second course in the resistant patients. Imatinib daily dose was 400 mg, then rose to 600 if tolerated. The CML patients received either interferon-based therapy (3) or hydroxyurea + cytarabine then were switched to Imatinib due to intolerance (400 mg) or progression to accelerated - blastic phase (ABP) (600 mg). Six patients started Glivec in remission or chronic phase (CP), four were resistant or in ABP. Four patients were in cytogenetic response, two also in molecular remission. Imatinib therapy was given for a median of 6 months (range: 2-18) prior to allogenic SCT. Seven patients (4 ALL and 3 CML) maintained or improved the degree of response, with two cases attaining a major cytogenetic response. Two ALL patients relapsed after 3.5 and 5 months, one CML patient lost cytogenetic response at twelve months and later progressed to AP. Three CML patients, one in late CP and two in ABP, were primarily resistant to Imatinib. Tolerance to Imatinib was generally good, with 4/10 patients developing grade III or higher toxicity (hematologic toxicity in two cases, hepatic in the other two). Imatinib therapy was stopped at a median of 1 month (range: 0.5-5) before transplantation. Allogenic SCT was performed in cytogenetic remission in three patients, CML-CP in one, ABP in four and relapse of ALL in two cases. Eight patients (80%) underwent transplantation from a matched unrelated donor (MUD), two from an HLA-identical sibling. Stem cell source was peripheral blood (PB) in 3/10 cases, bone marrow in 7/10. Median CD34+ dose was 3.9x10^6/kilograms (range: 0.5-6.4). Conditioning regimen included cyclophosphamide (CY) and TBI in eight patients, BUCY ± ATG in two. GVHD prophylaxis consisted of cyclosporine and short-course methotrexate. Nine out of ten patients engrafted, with a median time to neutrophil and platelet recovery of 19 (range: 13-29) and 22.5 (range: 17-130) days respectively. Two patients developed a grade III-IV acute GVHD (skin, liver, bowel) and died 27 and 55 days after SCT, the former still aplastic. Of the eight patients surviving more than three months, four (50%) developed a chronic GVHD, mild or moderate in three cases and severe in one, with cutaneous and pulmonary involvement. Three patient, who didn't suffered from cGVHD, relapsed after 3.5, 4 and 5.5 months: two patients died while the third attained remission after a second transplant. One CML patient, despite a moderate cGVHD, relapsed at molecular level after 7 months, but returned BCR/ABL-negative with Imatinib therapy. With a median follow up time of 16 months after SCT (range: 1-26+), 5/10 patients (1 ALL and 4 CML) are alive, in first (3) or second (2) remission. Three patients experienced major infections: two Gram-negative pneumonias and one HSV. One patient had CMV reactivation. Our experience confirms the safe-
ENGRAFTMENT, INCIDENCE OF GVHD (BOTH ACUTE AND CHRONIC) seem to affect the transplant procedure in terms of engraftment, incidence of GVHD (both acute and chronic) or infections.

PO-156
EXTRACORPOREAL PHOTOCHEMOTHERAPY: AN IMMUNOMODULATION APPROACH FOR ACUTE AND CHRONIC GVHD
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Acute and chronic GvHD strongly affect morbidity and mortality after allogeneic stem cell transplantation. The immunosuppressive therapy (IST) administered in order to control GvHD manifestations engravens on the post-transplant related infections and on the development of secondary malignancies. Furthermore IST strongly impairs the quality of life and growth in children. In this scenario, a new therapeutic approach based on immunomodulation rather than on a more aggressive IST is desirable. Extracorporeal Phototherapy (ECP) is a new therapeutic strategy consisting in a mononuclear cells (MNC) collection by a blood cell separator, dilution of the collected MNC with saline after 8-Methoxypsoralen addition, irradiation with UV-A light and the reinfusion of the manipulated MNC to the patient. This treatment has many advantages compared to other similar techniques: low extracorporeal volume (200 mL), short procedure time (2 hours), highly enriched MNC concentrate, homogeneous and efficient MNC irradiation, Hct less than 2% in the bag, a constant concentration of 8-MOP (2 ng/ml) in the leukapheresis product and, finally, low costs. We present our results about 91 patients (50 adults, 21 pediatrics) affected with grade II-IV chronic GvHD (Skin, mucosaes, liver and lung) and 20 pediatric patients affected with grade II-IV acute GvHD (Skin, mucosaes, liver, gut). ECP treatment schedule was: 2 consecutive procedures per week for 2 weeks, followed by 2 courses every two weeks for 3 times and finally by 2 procedures monthly. The response rate in adult and pediatric patients with cGvHD was 82% and 75% respectively, while in the pediatric setting with aGvHD the overall response rate was 62%. We were able to taper or suspend the IST in 79,3, 65,7% and 58% of adults and children with cGvHD and children with aGvHD respectively. ECP demonstrated its effectiveness as a second line therapy in the treatment of GvHD, its safety and tolerability was excellent. Finally, the possibility to decrease the IST, improved the quality of life, minimizing the long-term drugs related side effects. Even from an economical point of view ECP demonstrated to be a cost-effective therapeutical approach especially for long term survivals.

PO-157
EFFICACY OF SEQUENTIAL THERAPY WITH ANTI-CD20 (RITUXIMAB) AND PBSCT IN B LYMPHOPROLIFERATIVE DISORDERS: PHASE II STUDY
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A phase II study to valuate the efficacy of sequential therapy including purging with anti-CD20 monoclonal antibody (Rituximab) in conjunction with high dose therapy and PBSCT in lymphoproliferative disorders was performed. Treatment consisted of 4 CHOP, two mobilizations of CD34 cells with high-dose (HD) Cytoxan (CTX) + G-CSF and HD Aracytin (ARA-C) + G-CSF. Both therapies were followed by Rituximab as purging in vivo and PBSCT with BEAM conditioning regimen was planned as last phase. Values consisted at each step of bone and marrow biopsy with immune phenotype of cells and molecular biology of marrow sample; detection of purging efficiency with the same methods was also done. Up to December 03, 42 patients completed the entire program; 14 had been after relapse from previous treatment, 28 patients as up-front therapy. Histology included Follicular Center Cell Lymphoma (FCCL) in 17 pts, Diffuse Centroblastic Lymphoma (DBCL) in 2 pts, Lymphoplasmacytic Lymphoma (LPL) in 7 pts, mantle cell lymphoma (MCL) in 6 pts and chronic lymphocytic leukemia (CLL) in 10 pts. Twenty-two pts had leukemic syndrome. After the first phase with CHOP, all patients had a residual disease detected by immune phenotype or molecular biology. Following the first mobilization by CTX and first Rituximab 21 pts (50%) obtained a CR with absence of minimal residual disease. After the second mobilization by ARA-C and second Rituximab, 36 pts obtained a CR. Following the last phase with PBSCT 40 pts obtained CR. One patient died for Gram negative sepsis during cytopenia following PBSCT, one pt had cerebral hemorrhage and one fulminant hepatitis, 5 and 8 months after the end of program, respectively. Molecular remission was obtained in 85% of valuable pts. 32 pts remain in CR at a follow-up ranging from 8 to 50 months, mean 23 months. Results according to histology revealed a clinical and molecular remission in 93% of FCCL, a clinical and molecular CR of 60% and 20%, respectively, of CLL, a clinical remission of 40% of LPL, a clinical and molecular
remission of 100% of MCL and a clinical remission of 100% of CBL. In conclusion this study shows that sequential purging in vivo therapy with the combination of Rituximab and high dose therapy followed by PBSC CT is highly effective in inducing CR in most of CD 20 chronic lymphoproliferative disorders, however it seems that the efficacy is best expressed in FCCL and MCL.

PO-158

RELATIONSHIP BETWEEN CHIMERISM KINETICS OF LYMPHOPOIETIC SUBPOPULATIONS AND OUTCOME IN 35 PATIENTS RECEIVING ALLOGENIC NON-MYELOABLATIVE STEM CELL TRANSPLANTATION

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Chimerism analysis following allogeneic stem cell transplantation (SCT) is used to document clinical event such as engraftment, graft rejection or leukaemic relapse, but little is known about the establishment of chimerism in the different hematopoietic subpopulations in the setting of non-myoeloablative SCT. We have analysed chimerism in samples of peripheral blood, CD3+cell fraction and in bone marrow from 35 patients receiving allogeneic transplantation (between 04/99 and 02/04) from HLA identical sibling. Patients were conditioned with two different regimens of reduced-intensity conditioning: twenty-five patients received the association Fludarabine 60mg/kg, Cytoxan 60 mg/kg and Thiotepa 10 mg/m² (group A); then patients received the same dose of thiotepa plus cytoxan 100 mg/kg, but without the inclusion of fludarabine (group B). The median follow up was 300 days (20-1333); the median age of patients was 50 yrs (range 23-71), 16 were male and 19 female. All patients engrafted and the overall incidence of grade 3-4 acute GVHD was 14% and the non relapse mortality was 14%. The overall incidence of grade 3-4 acute GVHD was 14% and the non relapse mortality was 14%. The median follow up was 300 days (20-1333); the median age of patients was 50 yrs (range 23-71), 16 were male and 19 female. All patients engrafted and the overall incidence of grade 3-4 acute GVHD was 14% and the non relapse mortality was 14%. The evaluation of chimerism was performed in the bone marrow cells, in peripheral blood and in the following subpopulations: CD3+ cells and in mesenchymal cells; at days 15, 30, 60, 90 and 180 after transplantation, by using the PCR-based method for VNTR and STR microsatellites analysis; the CD3+ and CD56+ subpopulation analyzed have been obtained with Minimacs device while the mesenchymal cells (MSC) were analyzed after triple passage on Petri dishes of the adherent cells. The chimerism in the peripheral blood at day +30 was >95% in 22/25 (88%) patients of group A while only 5 out of the 9 patients conditioned without fludarabine achieved the complete chimerism; in the bone marrow the chimerism was complete in 16/22 group A patients (73%) and in 5/10 group B patients; the CD56 population evaluated in 14 patients showed a complete chimerism in all group A patients and in 3/5 group B patients. In the CD3+ population (evaluated in 23 patients) the chimerism was complete in 9/16 of those receiving fludarabine and in 1/7 of those conditioned without fludarabine. At day +90, in 23 evaluable patients the chimerism of PB and bone marrow remained complete in 15/20 group A patients while in only 3/8 (37%) of group B patients; also the CD3+ population in the fludarabine group showed complete chimerism in 88% (14/16) compared with 22% in the other group; the CD56+ population evaluated in 8 patients showed complete chimerism in all (7 conditioned with Fludarabine and one without fludarabine). Finally in the 10 patients evaluable for chimerism in MSC, 6/10 had no detectable donor cells while in 4 the percentage of MSC donor was ranging from 23 to 86%. Severe GVHD was observed in 5/35 patients, all conditioned with fludarabine; this suggests that the establishment of early complete chimerism in the lymphoid population could facilitate the onset of severe GVHD in this subset of patients.

PO-159

UNRELATED HEMATOPOIETIC STEM CELL TRANSPLANTATION IN THALASSEMIA: ROLE OF KIR POLYMORPHISM

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Several reports suggest that natural killer (NK) cells may be involved in rejection and GVHD following allogeneic bone marrow transplantation. Human NK cells express structurally diverse non-inhibitory and inhibitory receptors including the killer cell immuno-globulin-like receptors (KIR). These receptors are encoded by a family of 14 polymorphic genes and are characterized by the number of Ig domains (KIR2D or KIR3D) and by the length of their cytoplasmic tail, which can be long (L), with an inhibitory function, or short (S) with an activating function. Ligands of KIR are represented by HLA class I antigens at the A, B, Cw and G loci and are likely to play a significant role in the control of immune response and, probably, also in hematopoietic stem cell transplantation. In this study, we evaluated whether KIR genotype differences between HLA-identical donors and recipients had an impact on acute GVHD (grade II-IV) and rejection. 29 donor/recipient pairs, completely identical
Despite the relatively small size of the study in the course of bone marrow transplantation, genotype in the pathogenesis of GVHD and rejection was pertained to one of the other categories (3/11 pairs). However, none of these differences were significant. Of the 5 total cases of rejection, 4 pertained to the highest risk group (donor KIR genotype; in the remaining 11 donor/recipient pairs (38%) the KIR genotypes were different. When the donor KIR genotype was included in the recipient genotype, the incidence of GVHD was 30% (3/10 pairs). When the recipient KIR genotype was included in the donor genotype (high risk for GVHD), the incidence of GVHD was 67% (4/6 pairs). GVHD in recipients with different KIR genotypes was 27% (3/11 pairs). However, none of these differences were significant. Of the 5 total cases of rejection, 4 pertained to the highest risk group (donor KIR genotype included in the recipient genotype) and only one case pertained to one of the other categories (p = 0.03). Despite the relatively small size of the study, the results obtained suggest a possible role for the KIR genotype in the pathogenesis of GVHD and rejection in the course of bone marrow transplantation.

PO-160
ACUTE HEPATIC FAILURE AS ONSET OF PROGRESSIVE SCLERODERMATOUS CHRONIC GRAFT-VERSUS-HOST DISEASE AFTER DONOR LYMPHOCYTE INFUSION: A CASE REPORT
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Donor lymphocyte infusion (DLI) is an important therapeutic chance for the relapse post conventional allogeneic stem cell transplantation (SCT) and a therapeutic complement in non-myeloablative transplant. GVHD post- DLI still results in significant morbidity and mortality. A 28-year-old man with acute myelogenous leukemia (AML) underwent allogeneic SCT from his HLA-identical brother in July 2002, in first complete remission. The conditioning regimen consisted of busulfan and cyclophosphamide. Cyclosporin A (CyA) and short course methotrexate were given for GVHD prophylaxis. Acute GVHD was not observed. Relapse of AML was documented five months after SCT and CyA was discontinued. The disease was resistant to several salvage therapies and only Ara-C in continuous perfusion for 20 days was effective. In May 2003, the patient received the first (DLI) containing 2.5x10^7/kg of CD3. One month later a complete chimerism was evidenced in bone marrow and peripheral blood. The patient developed a grade III acute GVHD, involving skin and liver, responsive to methylprednisolone. A second DLI with 2.5x10^7/kg of CD3 was given in July 2003. Acute GVHD of grade I involving skin was evidenced on day + 21. Progressive chronic GVHD with lichenoid changes of mouth and liver involvement, confirmed by liver biopsy, developed from day + 41. The assessment of liver function showed increased serum transaminase, cholestasis enzyme and LDH (460 IU/L): ALP 312-158, AST 144, ALT 198 IU/L. Immunosuppressive therapy was not started to reduce the risk of relapse and in consequence of the non-severity of GVHD. 105 days after 2nd DLI, the patient acutely developed an anasarca-ic condition due to hepatic failure with decreased albumin (24 gr/l) and cholestasis (2721 IU/L), and increased prothrombin time (40 seconds). Transaminase and cholestatic enzyme were stable, except for an increase of ALP (420 IU/L); total bilirubin remained normal. RT-PCR for serum HCV, HBV, EBV, HHV, VZV, HSV and CMV antigenemia were negative. γ-globulin (IgG 2069 mg/dl polyclonal) and anti-nuclear antibody increased (1:2500). Ecography and doppler sonography didn’t demonstrate any alteration. A second liver biopsy was not conclusive because of an inadequate fixation of the sample. A therapy with albumin and diuretics i. v. was immediately started. The initial signs of sclerodermatous changes in the forearms and in the lower legs become evident with the reduction of the anasarca. The immunosuppressive therapy with CyA was given orally at dose of 1–2 mg/kg/days. Liver function normalized in three weeks with resolution of anasarca. Scleroderma remained stable until day + 145 after 2nd DLI; then it extended in the arms, legs, thorax and abdomen and diffuse joint contractions developed. Scleroderma wasn’t improved by the increased dose of CyA (3–5 mg/kg/day), the association of prednisone (1–2 mg/kg/day) and the following addition of methotrexate i. v. An acute hepatic failure evidenced an unusual flare of chronic GVHD. An autoimmune-like reaction inside a chronic GVHD probably acted on a liver which was damaged by antilastic therapies and emosiderosis, causing hepatic failure without a previous real acute hepatitis. Another interesting point is the relatively rapid resolution of anasarca and hepatic failure, immediately after the beginning of immunosuppressive therapy, against the progression of scleroderma. It can be hypothesized that a Th2-type reaction was the trigger of hepatic and sclerodermic flare; another hypothesis is that a Th2–response was followed by the shift toward a Th1–response. The first immunosuppressive therapy could have stopped Th2 reaction but not Th1 and/or the flow of cytokines and chemokines, which activate fibrosis through TGF–α. The characteristic course of GVHD post-DLI, similar to chronic GVHD but often with a development time typical of acute GVHD,
would require an early and intensive immunosuppressive therapy, specially in presence of scleroderma, but the beginning of the therapy is often retarded to reduce the risk of relapse. The decision about which cases require an immunosuppressive therapy and when it should be started is still an open question, specially post-DLI. An answer could come from a more precise knowledge of the immunologic phenomena involved. These phenomena seems to change not only with GVHD type (acute, chronic or post-DLI), but with time and target organs as well.

**PO-161**

**EVALUATION OF THE PROLIFERATIVE ACTIVITY OF HEMATOPOIETIC PROGENITORS FROM LEUCAPHERESIS IN DIFFERENT HEMATOLOGIC MALIGNANCIES**


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In 516 consecutive subjects, enrolled in a BMT program with peripheral blood progenitor cells (PBSC) and submitted to leucapheresis (LK) between January 1994 and april 2004, we studied the progenitor proliferative activity in each collection and the corresponding CD34+ cells percentage. The patients were affected by high-grade or resistant non-Hodgkin’s lymphoma (NHL, n=145), follicular non Hodgkin’s lymphoma (FNHL, n=29), Hodgkin’s disease (HD, n=79), multiple myeloma (MM, n=130), acute myeloid leukemia in first CR (AML, n=54), chronic myeloid leukemia in chronic phase (CML, n=54), chronic lymphocytic leukemia (CLL, n=4) idiopathic myelofibrosis (IM, n=5), AL amyloidosis (AA: n. 4); 45 healthy volunteers (VD) are also included in this series. The PBSC mobilizations were obtained with G-CSF after high dose chemotherapy (HD-CTX, IGEV or DHAP in HD, HD-CTX, IPAD or DHAP in NHL, R-HDara-C in FNHL, HD-CTX or D-CEP in MM, HD-Ara C in AML, IM, CLL, mini ICE in CML). G-CSF alone was used in AA and VD. The leucapheresis were performed with a CS3000 Plus (Baxter) or Spectra (Cobe), processing at least 2,5 blood volume per procedure. The collection started when CD34+ cell count was more than 20/µL and was stopped when CD34+ cells collected were more than 4×10^5/Kg. Independently from mobilizing chemotherapy, the LK number was not significantly different in all subjects analyzed, (mean 1.9 LK, p=0.16). Twenty% pts (n=106) required 3 collections, 5% (n=28) needed 4 and 2,4% (n=13) needed 5 or more leucapheresis. In NLH and HD patients the CFU-GM number and the percentage of CD34+ cells detected in the 1st LK were significantly higher than in 2nd (NHL: CFU-GM 263±360 vs 148±191×10^3 cells, % CD34 2.3±2.9 vs 1.3±1.4 cells; HD: 226±386 vs 89±00×10^3 cells, 1.7±2.1 vs 0.82±0.74). However, the CFU-GM growth, although does not reach statistical significance, decreased from the 1st to the last LK in all category of pts except in CML and MI pts when the progenitor number resulted progressively increased (CFU-GM/10^6 cells; CML 1st LK: 104±91;2nd LK: 131±90; IM 1st LK: 564±467, 2nd LK: 627±116). In conclusion the kinetic and the proliferative activity are similar in PBSC collected from NHL, HD, MM, AA pts and VD. A different growth pattern was shown by IM and CML cells, probably due to the peculiar biology of these disorders.

**PO-162** Not published

**PO-163**

**CHARACTERIZATION AND GENE EXPRESSION PROFILE OF A MESENCHIMAL STEM CELL CLONE**

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Human bone marrow contains at least two types of adult stem cells: hematopoietic stem cells (HSCs) and mesenchimal stem cells (MSCs). HSCs have been widely characterized and have been used in the clinical practice for decades. On the other hand MSCs have reached the scene only recently, nevertheless they are one of the most promising stem cell types for experimental and clinical applications. Several peculiar characteristics of this stem cell type make them so attractive: 1) they are easily accessible; 2) they are relatively easy to expand in culture; 3) they can be genetically manipulated; 4) they can differentiate in several cell types in vitro and in vivo; 5) they can transdifferetiate (i. e. differentiate in a cell type of different embryonic origin) in vitro and in vivo. Despite the appealing of MSCs as a therapeutic tool the knowledge of their biology is still very limited. An other drawback is the fact that there is no specific marker that identify unequivocally MSCs. Therefore MSCs have different characteristics in different laboratories and are probably a combination of cell types. Recently a multipotent cell has been clonally isolated from MSCs: the multipotent adult progeni-
tor Cells (MAPCs). MAPCs are able to differentiate in all the cell types of an organism when tested in an in vivo assay, recalling embryonic stem cells (ES) characteristics. In particular it has been shown that MAPCs participate to the erythroid compartment. MAPCs are valuable tool for gene therapy since their easiness of manipulation and the recent successful gene targeting by homologous recombination. In order to better understand the biological nature of MSCs (and possibly MAPCs) we are carrying out a gene expression profile on a single cell clone isolated from a population of MSCs. The single cell clone has been isolated from human bone marrow aspirate and has been expanded in culture in an undifferentiated state for more than 20 doubling. The clone has been characterized by immunocytochemistry being positive for several markers consistent with its undifferentiated state. We have also tested its capacity to transdifferentiate into glia and neurons. Total RNA has been extracted and a Sequence Analysis of Gene Expression (SAGE) has been carried out. A library has been constructed containing about 60,000 tags, representing the MSC transcriptome. Preliminary results indicate a complex pattern of gene expression, which will be further investigated.

PO-164
AUTOLOGOUS PERIPHERAL BLOOD STEM CELL TRANSPLANTATION FOLLOWED BY INTERLEUKIN-2 ADMINISTRATION IN ACUTE MYELOID LEUKEMIA PATIENTS IN IST COMPLETE REMISSION
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Interleukin-2 (IL2) is a cytokine with antitumor activity. When administered after autologous stem cell transplantation, it appears to reproduce the graft versus leukemia effect of allogeneic transplant and possibly prolong disease-free survival (DFS). Since 1999 at Hematology Department of Rome, 43 AML patients in Ist CR received PBSCT after BU-CY conditioning regimen. All patients received high-dose hydroxyurea followed by cytarabine, daunorubicin and etoside as induction treatment, and daunorubicin and high dose cytarabine as consolidation. In 28 out of 43 patients, a post-autograft immunotherapy with IL2 was planned. An absolute neutrophil count higher than 1,000/mL, a stable platelet count greater than 50,000/mL and no evidence of active infections were required to start the treatment. IL2 was administered subcutaneously on 5 consecutive days, on a monthly basis, for 1 year or until relapse. The dosage of IL2 was 4×10^6 IU on day 1, followed by 8×10^6 IU on days 2 through 5. All patients received during IL2 therapy paracetamol and prophylactic trimethoprim/sulfamethoxazole to prevent bacterial infections. Fifteen out of 28 patients were treated with IL2 after PBSCT. One patient is too early. One patient refused treatment and 11 were not eligible: 2 patients because of psychological disorders, 3 patients because of documentation of active infection after transplant, 2 patients who relapsed without PLTS recovery and 4 patients who showed a delayed platelet recovery at a median of 149 days (range 124–209) from transplant. Among the 15 evaluable patients, IL2 therapy was started after a median of 108 days from autograft (range 35–200). No patient had high grade toxicity requiring treatment discontinuation. All patients showed fever (38–39°C) with arthralgia (5/15 patients), 4–6 hours after IL2 administration. The majority of patients showed gastrointestinal toxicity (grade 1–2) in the form of nausea and vomiting (10/15), diarrhoea (4/15) and transient transaminase increase (2/15). Skin toxicity (grade 1–2) was observed as desquamation (7/15), rash and pruritus which required systemic measures (4/15) and injection site reactions (5/15). With regard to hematological toxicity (grade 2–3), transient neutropenia (2/15) and thrombocytopenia (4/15) were observed, requiring a 50% dose reduction in 2 patients. One patient showed irritability, during drug administration, not requiring dose modification. One patient showed herpes simplex infection requiring oral treatment. In all cases, toxicity completely recovered within 48 hours from IL2 discontinuation. Seven patients completed the treatment and are in CCR. Five patients are still on therapy. Three patients showed disease relapse after 2 (CNS relapse), 4 and 11 months of IL2 treatment, respectively. Our study shows that the IL2 therapy was often delayed after PBSCT because of a delayed stable platelet reconstitution. Based on our experience, it appears that low-dose IL2 after PBSCT is a feasible approach devoid of serious toxicity. A randomized trial is currently ongoing in the context of EORTC/GIMEMA AML12 protocol to document whether or not IL2 is capable to enhancing the likelihood of DFS after PBSCT.
Liver regeneration after tissue injury is dependent on two resident cell populations. Whereas moderate cell loss is restored by mature hepatocytes, more severe liver injury induces the activation of hepatic oval stem cells. Recently, the bone marrow has proven to be a third source of liver-repopulating cells. However, little is known regarding the mechanisms of mobilization of bone marrow stem cells into peripheral blood after liver injury, their concentration, phenotype and function. In the present study, peripheral blood after liver injury, their concentration, phenotype and function. In the present study, peripheral blood of 22 patients undergoing orthotopic liver transplantation (OLT) for liver failure and/or hepatocarcinoma and 6 patients submitted to partial hepatectomy (PH), were characterized phenotypically by flow-cytometry analysis. Five healthy subjects were used as control population. Hematopoietic (CD34, CD133, CD90, CD38) and endothelial markers (KDR, FLT-1, CD31, VE-Cadherin, vonWillebrand Factor) were monitored before (day -1) and after (days +1, 3, 7 and 14) the surgical procedure. A time-course evaluation of the colony forming activity of hematopoietic progenitors was also assessed in semisolid culture. Our results demonstrate that half of the OLT patients mobilized CD34+ hematopoietic progenitor cells into peripheral blood with a peak value at day +7 after OLT (0.23±0.30 cells/mm³ vs 1.95±2.71 cells/mm³; p 0.007). In addition, we found an increase of early CD34+CD90+ (0.11±0.16 cells/mm³ vs 0.51±0.72 cells/mm³) and CD34+/CXCR4+ progenitor/stem cells (0.21±0.38 cells/mm³ vs 1.18±1.46 cells/mm³; p 0.03) after 7 days from OLT. Clonogenic assay confirmed the mobilization of hematopoietic progenitor cells on day +7 and +14 from OLT and a positive correlation was observed between colony forming capacity and the number of CD34+ cells on day +7. Furthermore, the number of CD133+ (0.33±0.51 cells/mm³ vs 1.46±2.07 cells/mm³; p 0.006) and CD34+/KDR+ (0.03±0.1 cells/mm³ vs 0.39±0.75 cells/mm³; p 0.04) endothelial progenitor cells was also significantly increased on day +3 from OLT along with CD31+, VE-Cadherin+, and FLT-1+ mature endothelial cells. When we analyzed patients submitted to PH, we found a pattern of stem cell mobilization similar to that displayed by patients undergoing OLT. We also analyzed the serum levels of the following cytokines (SCF, HGF, SDF-1, IL-6) before and after 7 days from OLT: only the SCF serum levels were significantly increased on day +7 (1332±283 pg/ml) in comparison with the basal levels (1053±239 pg/ml; p 0.009). In conclusion, a significant proportion of patients submitted to OLT show a spontaneous mobilization of hematopoietic and endothelial progenitor cells into peripheral blood. Whether these mobilized cells participate to liver regeneration remains a matter of speculation and has to be determined further.

**PO-165**

**SPONTANEOUS MOBILIZATION OF BONE MARROW-DERIVED HEMATOPOIETIC AND ENDOTHELIAL PROGENITOR CELLS AFTER ORTHOTOPIC LIVER TRANSPLANTATION**

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Peg-filgrastim is a novel granulocyte-colony stimulating factor (G-CSF) characterized by prolonged activity and unique mechanisms of clearance. This drug is licensed in Italy for preventing and treating neutropenia after chemotherapy in solid and hematological malignancies. The efficacy of peg-filgrastim as mobilizing agent has not been extensively investigated. Here we report the case of a patient in whom peg-filgrastim was employed to induce mobilization of autologous peripheral blood stem cell (PBSC). A 62-year-old male patient affected by stage IIIA multiple myeloma was enrolled on January, 2004, in a front-line intensive chemotherapeutic program planned to perform tandem autologous stem cell transplantation. The patient was in partial remission after two cycles of DAV (doxorubicin, vincristine and dexamethasone) and had successfully collected 8.7×10⁶/kg CD34+ PBSC after one cycle with cyclophosphamide (4 g/m² body surface), followed by recombinant G-CSF at the dose of 10 mcg/kg s. c for 7 days. According to the protocol applied, a second administration of cyclophosphamide at the dose of 4 g/sqm was given two months after the first mobilization. Since the number of CD34+ cells previously collected was sufficient to perform both planned autologous transplantations, we decided, after informed consent was achieved, to use peg-filgrastim for the second mobilization. Forty-eight hours after cyclophosphamide, a single dose of peg-filgrastim (6 mg) was given subcutaneously. White
blood cell (WBC) nadir was reached on day +7 (1.7×10^9/L). Two consecutive leucaphereses were performed on day +9 and +10, when WBC counts were 8.6×10^9/L and 7.7×10^9/L and CD34+ cells were 0.3% and 0.2%, respectively. A total amount of 2.5×10^6/Kg CD34+ PBSC was collected. No relevant adverse effect were recorded. The patient referred only moderate bone pain at WBC recovery. The program of autologous transplantation is currently on-going. These data indicate that a single dose of peg-filgrastim is effective in mobilizing PBSC in combination with chemotherapy. However, in our patient, the total amount of CD34+ PBSC collected was lower than that previously obtained by using high dose G-CSF in the same setting. Optimal timing and doses and comparison with G-CSF, in terms of efficacy, safety and costs, warrant to be investigated in ad-hoc studies.

In CLL the primary event is the malignant transformation of CLL B cells. Defects of the residual cellular compartment (i.e. natural killer, T and dendritic cells), however, affect immune responses against neoplastic cells and lead to disease progression. Administration of cytotoxic cells such as natural killer (NK) and natural killer-like T (NKT) cells, expanded in vitro, may represent a novel opportunity in CLL treatment, but the difficulties found in ex vivo establishment of appropriate cytotoxic cells (CTL) recognizing the leukemic clone represent a limiting step in development of cellular immunotherapies. In an attempt to clarify expression of molecules involved in modulation of immune response, we here evaluated KIRs expression in a series of CLL patients, all with stable disease, at stage A and out of treatment. Among 16 patients studied, we could detect in 7 a significant expression of the p58.2 molecule (CD158b). Moreover in one patient we observed that the CD158b+ subset, representing half of the entire CD3+/CD8+ population, expressed a unique TCR Vβ region (Vβ23).

Cloning of purified CD19−/CD4+ cells, allowed us to isolate two TCR α/β (Vβ23+) CD3+/CD8+ and one TCR γδ (Vγ9/δ2) CD3+/CD8− T cell clones (TCC), all expressing p58.2. In functional studies we demonstrated that the addition of the anti-p58.2 mAb blocked cytotoxicity of these TCC (both TCR α/β and γδ) in CD3 redirected killing assay with P815. Furthermore, cytotoxicity of the gamma/delta TCC against 221 EBV cell line was inhibited by Cw4 but not Cw3 HLA expression on 221 cells. Altogether these findings suggest that expression of KIR with inhibitory activity by oligoclonal T cell expansions in B-CLL may contribute to immune evasion of leukemic B cells from T cell control.
TLR7 AND TLR9 LIGANDS EXHIBIT IMMUNOSTIMULATORY ACTIVITIES ON CHRONIC LYMPHOCYTIC LEUKEMIA B CELLS

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Natural and synthetic Toll-like Receptor (TLR) ligands can activate cells of the innate immune and some of them also show the ability to modulate immunocompetent cells. In particular, short-length CpG-containing motif oligodeoxynucleotides (CpG-ODNs) can stimulate proliferation and differentiation of murine and human B cells. More recently, imidazoquinolines have been demonstrated to stimulate normal human B cells through a TLR7 (and 8)–mediated signaling. Chronic lymphocytic leukemia (B-CLL) cells represent a mature malignant counterpart of B cells characterized by high apoptosis rate in vitro, unresponsiveness to ordinary agents promoting differentiation and proliferation. Limited data are known on TLR in B-CLL cells. In our study, the analysis of TLR1–10 by real-time PCR showed that highly purified CD5+ B-CLL cells expressed TLR7 and 9 whereas TLR8 and TLR1–5 were negative. We then compared the effects of TLR7–ligand R-848 (Resiquimod) with the phosphorothioate CpG ODNs DSP30 and 2006 which bind to TLR9. For control, LPS and synthetic double strand RNA poly I: poly C were used. R-848 was able to induce a significant proliferation of CD5+ leukemic B cells in almost all the analyzed B-CLL (90% cases) in a dose–curve manner with a similar rate of proliferation to CpG-ODNs. CFDA-SE analysis confirmed that CD19+CD5+ cells were responsible for thymidine-uptake. The stimulatory effect was directed to B cells as the depletion of PDCs did not affect the proliferative response. More interestingly, R-848 induced a strong up-regulation of costimulatory molecule expression (CD40, CD80, CD86) together with increased expression of CD20 and MHC class II molecules and R-848 and CpG-ODN pre-incubated B cells also exhibited increased stimulatory ability to allogeneic T cells in MLR even at very low E:R ratios (1:500). Finally, R-848 and, in a more limited way, CpG-ODNs were able to stimulate the differentiation of B-CLL cells into actively IgM-producing cells. Taken together, our data indicate that Resiquimod is a potent immunomodulator to induce maturation of neoplastic B cells to stimulate an efficient T cell response in some haematologic malignancies.

HIGH LEVELS OF ACTIVATED CASPASES IDENTIFY PATIENTS WITH INCREASED RISK OF DISEASE PROGRESSION IN B-CELL CHRONIC LYMPHOCYTIC LEUKEMIA


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B-cell chronic lymphocytic leukemia (B-CLL) is a disease characterized by the progressive accumulation of tumor cells which do not proliferate rapidly, but fail to undergo death. Even though almost all peripheral blood B-cells are arrested in G0 phase of the cell cycle, a proliferating pool of cells might be involved in disease progression. As a matter of fact, increased clonal expansion of leukemic B-CLL cells in progressive patients might be due to proliferation in excess of apoptosis. A central component of this apoptotic machinery is a proteolytic system that involves a family of proteases called caspases. These caspases can be divided into two groups: large prodomain containing upstream initiators (caspase-8, caspase-9), which may initiate the proteolytic cascade, and small prodomain containing downstream effectors (caspase-2, caspase-3, caspase-6), which in turn can amplify the signal by cleaving initiator-caspases and kill the cell by cleaving key intracellular targets. In order to define the prognostic impact of these apoptotic enzymes on the clinical outcome of B-CLL, we investigated 178 patients, median age 65 years, 88 males and 90 females. Activated caspases 2, 3, 6, 8 and 9 were determined on cellular cytosolic extracts through a spectrophotometric detection of the chromophore p-nitroanilide (pNA) after cleavage from the labeled substrates (VDVAD for caspase-2, DEVD for caspase-3, VEID for caspase-6, IETD for caspase-8 and LEHD for caspase-9). The pNA light emission was quantified using a microplate reader at 400–405 nm. Caspases activities were evaluated by the absorbance ratio samples/background readings (O. D. ). A mean value of all caspases activities (2, 3, 6, 8 and 9) was calculated for each B-CLL patient. The threshold of positivity was set at > 0.082 O. D. median value. With regard to patients characteristics, 53 had low Rai stage, 120 intermediate stage and 5 high stage. No significant correlations were found between modified Rai stages or β2-microglobulin and caspases activity levels. On the other hand, there was a very significant association between high caspases activity levels and lymphocyte doubling time (LDT) < 12 months (p=0.0004), confirming that a high proliferative rate was coupled with an excess of apoptosis. A slight correlation was found between low
The single VH gene most frequently encountered was VH3-72 (6/25; 24.0%), which, conversely, was very rare in nonselected (6/805; 0.74%; \( p<0.001 \)) or somatically mutated (5/432; 1.16%; \( p<0.001 \)) B-CLL and was exceptional among indolent (1/230; 0.43%; \( p<0.001 \)) and aggressive lymphomas (0/105; \( p<0.001 \)). Analysis of non-neoplastic B-cells revealed a very low frequency of VH3-72 rearrangements (less than 1%). VH3-72 genes of highly stable B-CLL disclosed a mutual hotspot at codon 84 of FR3 causing the substitution of isoleucine for threonine, whose frequency was significantly higher than in non-neoplastic B-cells (\( p<0.05 \)). Because this mutation substitutes a hydrophobic for a polar residue, it is expected to cause a conformational change of FR3 proximal to CDR3 that might critically affect high affinity antigen binding. All VH3-72 B-CLL used Vk family genes. Remarkably, 3 of 6 VH3-72 B-CLL utilized the same Vk gene B3, whose usage did not occur outside VH3-72 cases and appeared to be biased compared to nonselected B-CLL and normal B-cells. Cluster analysis revealed that two VH3-72 B-CLL had virtually identical VH CDR3 aminoacid sequences, differing only for conservative changes. Both cases rearranged the same D segment (D2-2, in the same reading frame) and an identical JH gene (JH3). The CDR3 of these 2 B-CLL had the same Vd usage, with a less biased D and J usage, would be compatible with a superantigen effect, no superantigen candidate has yet been linked to B-CLL. On the other hand, expansion of VH3-72 encoding B-cells may ensue stimulation by conventional antigens, as suggested by SHM in all VH3-72 B-CLL and significant caspases activity and high bcl-2 levels (\( p=0.05 \)). Finally, there was only a trend of association between low ZAP-70 or CD38 levels and low caspases activity (\( p=0.1 \), not significant). With regard to the clinical outcome, impressive shorter progression-free survival (PFS) and overall survival (OS) were observed in patients with higher caspases activity levels (18% vs 93% at 9 years; \( p=0.000001 \) and 74% vs 91% at 9 years; \( p=0.006 \), respectively). In multivariate analysis, caspases activity was confirmed to be an independent strong prognostic factor with regard to PFS (\( p=0.000001 \)) together with ZAP-70 (\( p=0.004 \)) and LDT (\( p=0.003 \)). Therefore, these apoptotic enzymes might represent a significant biological parameter useful to enucleate “high risk” B-CLL subsets. In conclusion, the considerable impact of apoptotic pathways on the clinical course of B-CLL patients are encouraging the experimental use of targeted-apoptosis drugs often in combination with chemotherapy in order to increase the response rate, but mainly to prolong time to progression and survival.

PO-170 HIGHLY STABLE B-CELL CHRONIC LYMPHOCYTIC LEUKEMIAS DISPLAY A BIASED USAGE OF IGV GENES AND HOMOLOGOUS IGHV AND IGLV CDR3S: IMPLICATIONS FOR ANTIGEN RECOGNITION IN LEUKEMOGENESIS

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B-cell chronic lymphocytic leukemia (B-CLL) may be dissected into two prognostic variants based on somatic hypermutation (SHM) of immunoglobulin variable (IgV) genes, whose presence contributes to predict for prolonged survival. Disease heterogeneity is further refined by identification of groups displaying a biased IgV use. Because a biased IgV usage may underlie a role of antigens/superantigens, recognition of B-CLL groups utilizing specific IgVH and/or IgVL genes may reinforce knowledge on leukemogenesis. Here, we explored IgVH and IgVL usage in highly stable and indolent B-CLL patients (n=25) who never required treatment over a 10-23 yr observation period. Data were compared to a total of 1,140 IgV genes from various B-cell malignancies: 805 nonselected B-CLL, of which 432 mutated and 373 unmutated, 230 indolent B-cell lymphomas and 105 aggressive B-cell lymphomas. SHM occurred in 24/25 productive VH rearrangements of highly stable B-CLL.
FR and/or CDR clustering of mutations in virtually all cases. Selection of FR3 aminoacidic substitutions and specific VH and VL CDR3 features, combined with a biased usage of VL genes, suggest that VH3-72 B-CLL express a highly homologous Ig structure recognizing a common epitope or, for cases with less conserved CDR3, various epitopes of the same antigen. Finally, because VH3-72 is virtually restricted to highly stable B-CLL, its detection may contribute to prognostic scores aimed at predicting for prolonged survival.

PO-171
MOLECULAR AND CLINICAL REMISSION CAN BE FREQUENTLY ATTAINED AFTER REDUCED INTENSITY CONDITIONING AND ALLOGENEIC TRANSPLANTATION, IN PATIENTS WITH RELAPSED CHRONIC LYMPHOCYTIC LEUKEMIA OR LOW-GRADE LYMPHOMAS


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Background: Graft-versus-leukemia effect (GVL) after allogeneic stem cell transplantation (allo-SCT) has already been described in chronic lymphocytic leukemia (CLL) and indolent lymphomas. Clinical responses can be achieved after cyclosporin withdrawal, donor lymphocyte infusions (DLI), and with the onset of graft-versus-host-disease (GVHD). In the present study we have evaluated the frequency of molecular remissions after reduced intensity conditioning (RIC) followed by allo-SCT. Methods: 40 patients with relapsed disease (22 CLL/SLL, 16 FCL, 1 MALT, 1 lymphoplasmacytic lymphoma) were enrolled in a prospective, multicenter phase II study. Median age was 55 years (range: 32-69). The median number of previous chemotherapy regimens was 3 (range 1-5) and 10 of 40 patients (25%) had already failed a previous autograft. Disease status at transplant was: chemorefractory disease in 32% of patients, partial or complete remission (CR=9; PR=17) in 62%; and minimal response in the remaining 6%. The conditioning regimen consisted of thiopeta 10 mg/kg, fludarabine 60 mg/ms and cyclophosphamide 60 mg/kg. GVHD prophylaxis consisted of short course methotrexate and cyclosporin 2 mg/Kg/die. Minimal residual disease (MRD) was monitored by nested PCR for IgH or Bcl-2 genes. In 4 PCR-positive patients (2 CLL and 2 FCL), a TaqMan based quantitative monitoring was also employed. For the two patients affected by CLL we designed a patient-specific IgH TaqMan system, whereas for the two FCLs we used a previously described TaqMan primer and probe set for Bcl-2 rearrangement. Results: All patients engrafted; 22 of 40 were in CR at day +30 after transplant. A molecular marker was found in 22 patients. Eighteen of 22 patients having a marker were alive: 10 of them attained the molecular remission. One patient died of transplant-related-mortality (GVHD and infection) while he was still in molecular remission. Seven patients never attained PCR negativity and 3 of them relapsed within a median time of 360 days from allo-SCT; one of them attained the molecular remission after chemotherapy and DLI, without developing GVHD. Eight of 11 patients who became PCR-negative between day +90 and +180 developed acute and/or chronic GVHD, supporting the hypothesis of a GVL effect. Two of the 4 patients monitored by quantitative PCR achieved the PCR negativity at day +30 and +90 after allo-SCT, while the other 2 patients were persistently MRD positive; in the latter patients the TaqMan PCR could detect an increase of tumour genomes in the marrow prior to the clinical relapse. Summary: RIC transplants can produce clinical and molecular remission. Quantitative PCR monitoring, can be used to tailor post-transplant immunotherapy.

PO-172
ROLE OF V61 T LYMPHOCYTES IN B-CLL PATIENTS: RECOGNITION OF MIC-A AND ULBP3 EXPRESSED BY LEUKEMIC B CELLS AND UPREGULATED BY ATRA


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In this study we analysed 38 patients with B-CLL (23 at low risk stage, 9 at intermediate risk stage, and 6 at high risk stage, according to the Rai modified classification), chemotherapy naïve. Interestingly, circulating Vdelta1 T lymphocytes were increased in 15/38 patients, all at low risk; once isolated from peripheral blood Vdelta1 T cells proliferated in vitro...
and produced TNFα or IFNγ in response to autologous B cells. These γδ T cells were cytolytic against the murine mastocytoma P815, upon engagement of either TCR or NKG2D with the specific monoclonal antibodies, suggesting that they have an intact cytolytic function. However, γδ T lymphocytes did not kill resting autologous tumor B cells, which lacked the MHC-related MIC-A antigen and expressed low levels of the UL16-binding-proteins (ULBP) 1 and 3 and undetectable ULBP2 and 4. These molecules are all reported ligands for γδ T cells that recognize them via the NKG2D receptor. Indeed, the Vδ1 T cell subset could lyse the C1R B-cell line transfected with MIC-A or autologous B-CLL when transcription and expression of MIC-A and ULBP1 or upregulation of ULBP3 was achieved upon exposure to trans-retinoic acid (ATRA). Moreover, NKG2D was expressed on Vδ1 T cells involved in the recognition of B-CLL on which MIC-A or ULBPs were induced by ATRA. Interestingly, in six patients displaying a low number of circulating Vδ1 T cells, and undetectable ULBPs, the disease progressed in the last year, at variance with patients with high Vδ1 T lymphocytes and detectable/inducible ULBP3.

**PO-173**

**DISTINCT NUMBERS OF D13S319 AND RB1 ALLELES IN PATIENTS WITH B-CELL CHRONIC LYMPHOCYTIC LEUKEMIA REVEALED BY FLUORESCENT IN SITU HYBRIDIZATION**


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In B-CLL bands 13q12-14 are frequently targeted by interstitial/terminal deletions and therefore are supposed to contain an as yet uncharacterised tumour suppressor gene. The aims of the present study were to establish whether subclones with distinct numbers of D13S319 and RB1 alleles could be simultaneously present in B-CLL patients and whether they are correlated with any peculiar clinical parameter and disease outcome. FISH was carried out either on mitotic cells or on interphase nuclei obtained from seventy-one patients. It was performed with two probes, i.e. LSI13 and D13S319, both commercially obtained from Vysis (Downers Grove, IL, USA) and simultaneously applied according to manufacturer’s guidelines. A normal cellular pattern was defined by the presence of two green and two red signals, while a cell harbouring a deletion/nullisomy was defined by either three or two signals respectively. No patient presented a nullisomy for both the loci examined. This result was per se sufficient to document effective hybridisation and allowed us to set the cut-off values at 6% for interphase FISH. This last value was determined by analysing two hundred nuclei obtained from ten normal controls and by correcting the mean percentage obtained for three times the standard deviation. An abnormal FISH pattern was discovered in 42-500 from 30 patients (42.2%). The deletion of one D13S319 locus and of one RB1 allele was seen in nine patients, a cell line with the same pattern (one D13S319 locus and one RB1 allele deleted) along with another one with the deletion of only one D13S319 locus in two patients, the deletion of one D13S319 locus in ten patients, the deletion of both D13S319 loci in two patients, a complex pattern in five patients, the deletion of both D13S319 loci along with the deletion of one RB1 allele in one patient and the deletion of one RB1 allele in one patient. Patients with a complex pattern presented the most interesting FISH results. Three of them showed two cell lines one with the loss of one D13S319 locus and the other with the nullisomy of the same locus. This pattern was observed in more than two hundred cells. The remaining two patients with a complex pattern harboured two cell lines one with the deletion of one D13S319 locus and the other with the nullisomy of the same locus and the deletion of one RB1 allele. This last pattern was discovered in more than one hundred cells. Most of our patients had been investigated on clinical diagnosis and were in an early disease stage. In contrast, patients with the monosomy of both the loci were predominantly in advanced disease stage. According to Binet three patients were classified as stage B and three as stage C; according to Rai two patients were classified as stage two, two as stage three and one as stage four. Three patients experienced clinical progression. A monoclonal protein was detected in three patients with the deletion of the D13S319 allele. In conclusion the observation that B-CLL patients may simultaneously harbour multiple cell lines with different numbers of D13S319 and RB1 alleles suggests that 13q deletion is probably produced by chromosome evolution within the neoplastic clones. This suggestion is reinforced by the fact that a spontaneous conversion to a normal FISH pattern occurred in two of our patients who presented the deletion of one D13S319 allele on clinical diagnosis. In addition, patients with the monosomy of band 13q14 more frequently have an advanced disease and more often experience disease evolution.
**PO-174**

**INDUCTION OF FAS UPREGULATION DOES NOT RENDER CHRONIC LYMPHOCYTIC LEUKEMIA B CELLS SUSCEPTIBLE TO FAS-MEDIATED APOPTOSIS**

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Chronic lymphocytic leukemia (CLL) is characterized by a progressive accumulation of long-lived and well-differentiated clonal B lymphocytes in the peripheral blood, lymphoid tissues and bone marrow. Although CLL pathogenesis is not entirely understood, the progressive increase in lymphocyte counts coupled with very low proportion of proliferating cells suggests that this disease may be primarily determined by defective apoptosis. Consistently, freshly analyzed CLL B cells express very low levels of membrane Fas (APO-1, CD95), one of the best known receptors involved in triggering the apoptotic machinery. In the attempt of exploring new approaches for immunotherapy of CLL, the aims of the present study were: i) to work out means to increase Fas expression on CLL B cells; and ii) to assess whether Fas-expressing CLL B cells could be induced to undergo apoptosis following Fas stimulation. Fas upregulation on CLL B cells was induced by coculturing clonal B cells with preactivated autologous T lymphocytes or their supernatants. Intracellular cytokine staining of preactivated autologous T lymphocytes or their supernatants. Blocking experiments using moAbs specific for IL-2 and IFN-γ, showed these cells to contain mainly IL-2 and IFN-γ. Blocking experiments using moAbs specific for IL-2 or IFN-γ revealed that the Fas-enhancing activity in T cell supernatants was mainly due to IFN-γ. However, following stimulation with an agonistic anti-Fas moAb or recombinant human soluble FasL for up to 72 h, Fas-expressing CLL B cells were found to be resistant to Fas-mediated apoptosis, as assessed by flow cytometry evaluation of annexin V-binding and propidium iodide staining, confirming the notion that altered apoptosis plays a relevant role in the pathogenesis of this disease and showing that this phenomenon was not due to reduced Fas expression. Finally, immunoblot experiments showed that CLL B cell resistance to apoptosis was not associated with lack of caspase-3, as clonal B cells expressed similar levels of this protein as Jurkat T cells, which were used as a positive control in all apoptosis experiments. Further research is needed to identify the molecular mechanisms underlying apoptosis resistance in CLL.

**PO-175**

**MOLECULAR ANALYSIS OF IMMUNOGLOBULIN HEAVY CHAIN REARRANGEMENTS AND VH SOMATIC MUTATIONS IN LYMPHOPROLIFERATIVE DISORDERS USING LASER CAPTURE MICRODISSECTION**

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Laser Capture Microdissection (LCM) is a powerful technique capable of obtaining target single cells without contamination from surrounding populations from histologic and immunostained sections. Combining this new approach and the polymerase chain reaction (PCR), clonally rearranged Immunoglobulin heavy (IgH) chain genes are reliably amplifiable in isolated lymphoid cells. Moreover, IgH sequencing analysis provides a rapid and helpful method to establish correlation between genetic, morphology and immunophenotype, even at the single cell level. The degree of somatic hypermutations also improves the study of the stage of differentiation of cells involved in the lymphoproliferative disorders. Trying to evaluate the capability of this novel technique on complex and heterogeneous lymphoma tissues, we studied a case of a 64-year-old man, affected by Chronic Lymphocytic Leukemia (CLL) with diffuse lymphadenopathy, in which the presence of Reed-Sternberg (R-S) cells raised the possibility of a coexistent Hodgkin Disease. In fact, the histological evaluation showed a Small Lymphocytic Lymphoma (SLL) with large proliferation centers carrying a great amount of immunoblasts and isolated R-S cells. Immunohistochemical staining demonstrated that both small lymphocytic and proliferation centers expressed CD5, CD20, CD43; the latter were also partially CD30-positive. R-S-like cells expressed CD15 and CD30 but were CD5 and CD20-negative. Formalin fixed hematoxilin-eosin stained sections and frozen sections immunostained for CD30 were micro-manipulated using LCM (Leica DM LMD): single CD30-positive cells and multiple cells samples from small lymphocytic and proliferation centers were collected. A semi-nested strategy was used for the PCR amplification of the IgH chain gene using consensus primers complementary to the conserved framework-2 segment of the variable (V) region and to the joining (J) region. PCR products were gel-purified and directly sequenced in both directions on an auto-
mated capillary system (AB310). The sequences were compared with published germ line data to identify V-D-J rearrangements and VH somatic mutations. The results of the IgH sequence analysis showed the presence of a common predominant clone (VH3-66/DH3-3/JH6) in SLL areas, in proliferation centers and in R-S-like cells. This rearrangement was the same found in the whole section of the lymph node, supporting a clonal relationship between CLL/SLL and R-S-like cells. In addiction none of the rearrangements examined carried VH somatic mutation supporting the naïve nature of the lymphomatous components. Therefore, LCM followed by PCR and sequencing techniques, provides an important tool for the investigation of Ig status and the clonal relationship in purified cells from lymphoma tissues.

**PO-176**

**CONVENTIONAL CYTOGENETIC ANALYSIS AND INTERPHASE IN SITU HYBRIDIZATION IN 28 CASES OF DLBCL**


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Diffuse large-cell lymphoma (DLBCL) is a histologically well defined subset of non-Hodgkin lymphomas which comprises several entities characterized by different genetic, immunophenotypic and clinical features. For example, only 45% of patients achieve a complete remission, while the remaining patients dead of the disease, despite treatment. Moreover, DLBCL may arise de novo or may be an evolution of previous low grade lymphomas. For all these reasons DLBCLs result an heterogeneous disease and so far very few genetic and biological markers are available to predict the behaviour of these lymphomas. Approximately 50% of DLBCL exhibit chromosomal translocations involving Ig heavy chain genes, located on 14q32 region, and different partners. The most frequently involved partner gene is BCL2 gene. In addition none of the rearrangements examined carried VH somatic mutation supporting the naïve nature of the lymphomatous components. Therefore, LCM followed by PCR and sequencing techniques, provides an important tool for the investigation of Ig status and the clonal relationship in purified cells from lymphoma tissues.

**PO-177**

**INTERNATIONAL SURVEY OF PRIMARY EFFUSION LYMPHOMA (PEL)**


PEL is a rare B-cell neoplasm characterized by a preferential involvement of fluid-filled body spaces, consistent infection of the tumor clone by human herpesvirus type-8 (HHV-8) and a close relationship with underlying immunodeficiency status of the host. The International Extranodal Lymphoma Study Group (IELSG) coordinated a retrospective survey involving 14 international institutions to determine the clinical-pathological features and patterns of outcome of PEL. Forty-two patients (37 males and 5 females) were registered. Median age at diagnosis was 58 years (range 27-102). In 23 (55%) patients an associated human immunodeficiency virus (HIV) infection was reported, in one case the diagnosis of PEL was made after a solid organ transplantation, in two patients other immunodeficiency conditions were present. The HHV-8 infection of the tumor clone was demonstrated in 34 out of the 38 tested cases, Epstein-Barr virus infection in 13 of 29 cases. CD4 count was lower than 200/µL in 18 of the 25 cases in whom the data was available. An ECOG performance status score 2 was observed in 28 patients and the presence of B-symptoms in 20 patients. Serum LDH was elevated in 20 of the 38 tested patients. In 4 patients nodal involvement at diagnosis was reported, in 4 cases at least one extranodal site of localization other than serous cavities was present. A low/low-intermediate risk score according to International Prognostic Index was reported in 10 cases, an intermediate–high/high risk score in 28 cases. Twenty patients received systemic chemotherapy, in 16 cases an anthracycline-based regimen. Intraperitoneal cidofovir was administered in 3 patients. Twelve HIV+ patients received highly active retroviral therapy (HAART), four of them as single therapy. xAmong the 38 patients for whom adequate follow-
up data were available, median overall survival was 5 months, median cause-specific survival was 12 months and median progression-free survival was 8 months. Interestingly, cases of tumor complete regression after implementation of the sole HAART, after intrapleural administration of cidofovir or without any treatment were reported. Our data confirm the poor prognosis of PEL but suggest a possible heterogeneity of this entity with respect to its biological and clinical features. A review of the pathological, phenotypical and virological features is forthcoming to validate these preliminary results.

PO-178
LONG TERM MONITORING OF CANCER-FREE SUBJECTS CARRYING NON-LYMPHOMA ASSOCIATED BCL2/IGH REARRANGEMENTS (NLABR): PROLONGED PERSISTENCE OF CLONAL POPULATIONS POTENTIALLY RELATED TO FL
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Introduction: NLABR are frequently observed in cancer-free subjects. We have recently observed that cells NLABR-positive clones can persist up to 60 days (Ladetto et al., J Clin Oncol 2003). However the long-term kinetics and potential pre-neoplastic role of cells carrying these rearrangements is unknown. Aim of this study was to perform a long term monitoring of NLABR-positive subjects in order to define the natural history of NLABR-positive clones. Methods: 14 NLABR-positive subjects undergoing periodical blood examinations for warfarin therapy have been monitored for a median time of 12 months (range 3–24 months). NLABR-positive clones have been analyzed using both nested and real time-PCR according to standard procedures (Ladetto et al. Exp Hematol 2001). Sequence homology of NLABRs has always been confirmed by direct sequencing of both forward and reverse DNA strands of nested PCR products. Results: in seven subjects (50%) prompt disappearance of PCR positivity was noticed without reappearance of the rearrangement. This seven subjects have been monitored for median period of 12 months (range 3–18 months). An unrelated rearrangement was detected during subsequent follow-up in one of these subjects. In the other seven subjects (50%) the same rearrangement observed at study initiation has been detected one or more times on follow-up evaluations. In four subjects with persistent NLABRs the rearrangement detected at diagnosis was consistently detected in all follow-up samples, while in three the NLABR detected at diagnosis could be amplified only in a fraction of follow-up samples while the remaining turned out to be PCR-negative. Overall, persistent NLABRs could be followed on these subjects for a median time of 15 months (range 3–28). The median burden of persistent NLABR-positive clones assessed by real time PCR was 33 rearrangements/106 diploid genomes (dg) (range <10–760), while the median burden of short-lived NLABR-positive clones was <10 rearrangements/106 dg (range <10–330). The number of NLABR-positive cells appeared to be rather stable in all of these subjects: in none of them we could detect differences greater than 1 log among available follow-up samples. Discussion NLABR-positive clones are long-lived cell populations in at least 50% of cases. Based on this finding it is reasonable to hypothesize the existence of a follicular lymphoma (FL)-related lymphoproliferation of undetermined significance. Since the average incidence of NLABR in previously published series is always greater than 10%, this condition is expected to be frequent in the general population (as observed in MGUS and CLUS) and might be of relevance for the pathogenesis of FL.

PO-179
ABERRANT SOMATIC HYPERMUTATION IS INVOLVED IN PRIMARY MEDIASTINAL LARGE B-CELL LYMPHOMA AND PREFERENTIALY AFFECTS THE PAX-5 GENE
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Primary mediastinal large B-cell lymphoma (PMLBCL) is recognised as a subtype of diffuse large B-cell lymphoma (DLBCL) arising in the mediastinum. With respect to DLBCL, PMLBCL shares specific clinical, morphological and molecular features suggesting that PMLBCL may represent a distinct clinico-pathologic entity. This concept has been recently reinforced by the notion that DLBCL and PMLBCL display different gene expression profiles. Aberrant somatic hypermutation of multiple proto-oncogenes relevant to lymphomagenesis, namely PIM-1, PAX-5, RhoH/TTF and c-MYC, has been advocated as a molecular fea-
ture distinctive of DLBCL. To investigate whether the same mechanism is associated with PMLBCL, we performed mutational analysis of PIM-1, PAX-5, RhoH/TTF and c-MYC in a panel of 9 PMLBCL. For each gene, a region spanning up to 1.5 Kb from the transcription start site and previously shown to contain >90% of mutations in B-cell lymphoma was analysed by PCR amplification and DNA direct sequencing. Mutations targeting at least one of the 4 genes were found in 5/9 (55.5%) PMLBCL, while mutations in more than one gene were found only in 1/9 (11.1%) cases. Among the four genes tested, only PAX-5 was altered in a significant fraction of cases, since it was mutated in 4/9 (44.4%) PMLBCL. In contrast, PIM-1 was mutated in 1/9 (11.1%), RhoH/TTF was mutated in 1/9 (11.1%) and c-MYC was mutated in 1/9 (11.1%) PMLBCL. A total of 29 mutational events were detected in 9 PMLBCL. The overwhelming majority of mutations affected PAX-5 (n=24). The mutation frequency for PAX-5, calculated only on mutated cases, was 0.36±0.2-2/bp and was 2.25 to 9 fold higher than that occurring in PIM-1 (0.16±0.2-2/bp), RhoH/TTF (0.06±0.2-2/bp), c-MYC (0.04±10-2/bp). The majority of the mutations were represented by single base-pair substitution (n=26). Two PMLBCL carried 3 deletions of a short DNA stretch (2–21 bp) affecting PAX-5. Of the 26 single base-pair substitutions, eleven were transitions and 15 were transversions, with a transition/transversion ratio of 0.78 (expected 0.5). These results indicate that, among germinal center related lymphomas, PMLBCL seems to display a distinct profile of aberrant somatic hypermutation. In fact, whereas DLBCL is affected by aberrant somatic hypermutation of the PIM-1, PAX-5, RhoH/TTF and c-MYC genes in nearly half of the cases, aberrant somatic hypermutation in PMLBCL targets preferentially the PAX-5 gene, sparing PIM-1, RhoH/TTF and c-MYC. Interestingly, 50–70% PMLBCL carry chromosomal gains of 9p, that represent the most frequent cytogenetic lesion in these lymphomas. Since PAX-5 is located at 9p13, amplifications and mutations of the PAX-5 locus may cooperate in lymphomagenesis in the context of PMLBCL. Because PAX-5 mutations occur in the regulatory region of the gene and may affect transcription, these alterations may cause deregulated expression of PAX-5, that is a key gene in the control of B-cell differentiation.

High-dose methotrexate (HD-MTX; /1 g/m²) is the most effective drug against primary central nervous system lymphomas (PCNSL). The major route for cellular uptake of MTX involves the reduced folate carrier (RFC), a bi-directional anion transporter with high affinity for reduced folates and antifolates. Defective transport via the RFC may be a common mechanism of resistance to antifolates. Studies in patients with acute lymphoblastic leukemia and osteosarcoma have suggested that defective transport via the RFC may be a common mechanism of resistance to antifolates. Lack of RFC expression in MTX-resistant human tumor cell lines has been ascribed to the aberrant methylation of RFC gene promoter, since MTX-sensitive human tumor cell lines are devoid of this epigenetic abnormality. The aim of this study was to investigate the prevalence of aberrant methylation of RFC promoter in PCNSL in immunocompetent patients and to correlate the methylation status of RFC to MTX resistance and therapeutic outcome. Genomic DNA from biopsy specimens of 40 PCNSL in immunocompetent patients was used as a template for methylation–specific PCR (MSP) and bisulfite genomic sequencing (BGS) of RFC promoter. Fifty HIV-negative cases of systemic diffuse large B-cell lymphomas (DLBCL) were used as controls. MSP showed evidence of aberrant methylation of the RFC gene in 12 of 40 (30%) PCNSL samples and in 4 of 50 (8%) DLCL used as controls (p=0.01). To further define the methylation status of RFC promoter, representative
samples showing RFC methylation by the MSP assay were further analyzed by BGS. All cases tested displayed methylation in virtually all cytosines of the CpG dinucleotides localized in the CpG island. Analysis of RFC mRNA expression was performed by RT-PCR in representative methylated and unmethylated cases. Methylated samples demonstrated absent or markedly low levels of RFC transcript when compared to unmethylated samples or normal controls. Because infiltrating normal cells are generally present in tumor samples, this low level of expression in a fraction of methylated tumors may reflect transcription from unmethylated normal cells. Impact on outcome of RFC promoter methylation was assessed in 37 PCNSL patients who were treated with HD-MTX-based chemotherapy ± radiotherapy. Among the 37 PCNSL patients treated with HD-MTX-based chemotherapy, RFC promoter methylation was detected in 9 cases (24%), M-PCNSL, while 28 (76%), U-PCNSL scored negative. Three patients with M-PCNSL (33%) and 15 with U-PCNSL (54%) achieved a complete remission after primary chemotherapy. Logistic regression confirmed a near significant association between complete remission rate and RFC promoter methylation (p = 0.07). Moreover, RFC promoter methylation was related to a worse failure-free survival with overall survival. Although difference in survival of patients with M-PCNSL or U-PCNSL did not reach significant levels, none of M-PCNSL cases were relapse-free at 3 years, and all 3-year survivors had U-PCNSL. Implications of these data are multifold. First, this is the first study analyzing the methylation status RFC gene promoter in human tumor samples and demonstrating the presence of this epigenetic alteration in PCNSL and DLBCL from untreated patients. Second, the high frequency of RFC promoter methylation (30%) in PCNSL and the near significant association between this epigenetic alteration and complete remission rate, put this epigenetic mechanism among those potentially involved in intrinsic MTX resistance in PCNSL patients. Consequently, our findings suggest that the analysis of RFC methylation status could allow us to distinguish PCNSL patients treated with HD-MTX-based chemotherapy in subgroups with different outcome on the bases of the methylation status of RFC gene. Finally, analysis of RFC promoter methylation might have potential role in therapeutic choice and investigation of novel strategies. For instance, MTX transport impairment may lead to study newer antifolates like trimetrexate, which does not depend on RFC for cell entry. Alternatively, demethylating agents, like 5-aza-2'-deoxycytidine, could be used to restore RFC activity, reverting MTX resistance.

**PO-181**

**THE HLA-DR-SPECIFIC MONOCLONAL ANTIBODY 1D09C3 EXERTS A POTENT ANTITUMOR ACTIVITY ON MALIGNANT LYMPHOID CELLS BOTH IN VITRO AND IN VIVO**


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Significant proportions of patients with lymphoproliferative disorders, including non-Hodgkin lymphoma (NHL), relapsed Hodgkin’s lymphoma (HL), and chronic lymphocytic leukemia (CLL) are not cured with currently available therapeutic strategies. Additionally, due to age restrictions, substantial proportions of these patients are not eligible for stem cell transplantation. Therefore, new treatments targeted to the malignant clone are needed. The human leukocyte antigen (HLA)-DR is one of three highly polymorphic genes of the class II major histocompatibility complex, which, under normal conditions, are selectively expressed on cells of the immune system. A fully human antibody of IgG4 isotype, called 1D09C3, targeting HLA-DR has recently been generated. It, therefore, was the aim of the present study to investigate the in vitro and in vivo activity of 1D09C3 on a large panel of lymphoma cell lines, including HL (L1236, L428, L540, HDMY-Z, HDLM-2, KM-H2), CLL (JVM-2, JVM-3, JVM-13, MEC-1, MEC-3, EHEB), and NHL cell lines (GRANTA-519, KARPAS-299, SUP-M2). To evaluate the in vitro activity of 1D09C3, lymphoma cells (5 × 10⁶/mL) were exposed to 1D09C3 (2.5 µg/mL, 48 hours). At the end of the incubation, viable cell counting was performed by FACS using Flowcount beads, cell survival was evaluated by the MTT assay, and apoptosis was evaluated by annexin-V expression. As compared to controls, exposure to 1D09C3 significantly (0.01) reduced the mean (±SEM) viable cell counts to 30±8%, 33±8%, and 14±4% for HL, CLL, and NHL cell lines, respectively. The same figures for cell survival as evaluated by the MTT assay were 28±7%, 26±9%, and 34±11%, respectively. Analysis of annexin-V expression revealed significant increases of the percentages of apoptotic cells in 1D09C3–treated cultures as compared to controls (HL: 64±8 vs 20±5, P<0.004; 003; CLL: 33±2 vs 13±2, 0.001; NHL: 53±11 vs 16±4, 0.01). Interestingly, 1D09C3 (2.5 - 10 µg/mL) had no toxic effect on CD34+ cells, suggesting that the antitumor activity of this antibody is restricted to HLA-DR+ lymphoma cells. The in vivo activity of 1D09C3 was evaluated in a xenotransplant model of human lymphoma in nonobese diabetic/severe combined immunodeficient nonobese diabetic/severe combined immunodeficient nonobese diabetic/severe combined immunodeficient nonobese diabetic/severe combined immunodeficient nonobese diabetic/severe combined immunodeficient nonobese diabetic/severe combined immunodeficient nonobese diabetic/severe combined immunodeficient nonobese diabetic/severe combined immunodeficient nonobese diabetic/severe combined immunodeficient nonobese diabetic/severe combined immunodeficient nonobese diabetic/severe combined immunodeficient nonobese diabetic/severe combined immunodeficient nonobese diabetic/severe combined immunodeficient nonobese diabetic/severe combined immunodeficient nonobese diabetic/severe combined immunodeficient nonobese diabetic/severe combined immunodeficient nonobese diabetic/severe combined immunodeficient nonobese diabetic/severe combined immunodeficient nonobese 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cient (NOD/SCID) mice. Mice were inoculated intra-
peritoneally with JVM-2 cells (2.5–10^5 per mice) and were treated with 1D09C3 (3×1 mg/mouse, sub-
cutaneously) starting either on day 5 (early stage lymphoma) or on day 14 (disseminated stage lymphoma). Endpoint was mice survival. 1D09C3-treated mice were compared to placebo-treated NOD/SCID mice. All placebo-treated mice transplanted with 2.5×10^5 (n = 15) and 10×10^5 (n = 15) cells/mouse died after a median survival of 35 and 29 days, respectively. In contrast, all mice transplanted with 2.5×10^5 JVM-2 cells/mouse and treated with 1D09C3 at an early stage of disease were alive at +120 days with no evidence of disease (p=0.0001). Mice transplanted with 10×10^6 JVM-2 cells/mouse and treated with 1D09C3 showed a median survival of 99 days and 42% of them were alive at +120 days with no evidence of disease (p=0.005). Mice treated for a disseminated stage lymphoma showed a significant survival advantage as compared to controls (52 vs 29 days, p=0.0001). No mice experienced any apparent treatment-related toxicity. In conclusion, 1D09C3 has no toxic effect on CD34+ cells, whereas it has a strong antitumor activity both in vitro and in vivo on HLA-DR-positive lymphoma cell lines. Such monoclonal antibody offers the potential for a novel therapeutic approach to lymphoid malignancies.

Primary effusion lymphomas (PEL) are rare forms of B-cell malignancy that show a peculiar resistance to conventional pharmacologic drugs and a strikingly adverse prognosis. In this study, cancer testis antigens (CTA) were investigated as potential immunotherapeutic targets in patients with PEL. Baseline expression of a panel of 11 CTA was highly heterogeneous among 5 PEL cell lines. In particular, the investigated CTA were not expressed in BC-2 and BC-3 cells, while BC-1, HBL-6 and BCBL-1 cells tested positive for 6, 8 and 9 CTA, respectively. The DNA hypomethylating agent 5-aza-2’-deoxycytidine (5-AZA-CdR) invariably induced or up-regulated the expression of all investigated CTA in all cell lines analyzed. The de novo expression of CTA was still detectable at mRNA and protein level at least 2 months after the end of 5-AZA-CdR treatment. These findings, and the concomitant up-regulation of HLA-class I antigens and ICAM-1 by 5-AZA-CdR, support its clinical use to set-up innovative chemo-immunotherapeutic approaches in PEL.

**PO-183**
**EX VIVO ELISPOT MAY BE A NEW TOOL FOR THE EARLY DIAGNOSIS OF MYCOBACTERIAL INFECTION IN HEMATOLOGIC PATIENTS**

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In the last few years there has been an increasing number of reports on Mycobacterial infections (MBI) in hematologic patients. Hairy cell leukemia (HCL) and stem cell transplant (SCT) patients are the much more affected categories, with incidence ranging from 1% to 9%, but the problem appears to be underestimated. Despite the availability of new molecular biology methods, the diagnosis of MBI is often delayed with obvious clinical consequences. Tools for MBI diagnosis, such as isolation of mycobacteria through acid-fast staining, cultural examination, tuberculin skin test (TST) and ligase chain reaction, are limited by low sensitivity and specificity, especially in immunocompromised host (IH), resulting often falsely negative, and are time consuming. Consistent with this, a recent report has shown that in hematologic patients the mean time interval between first symptoms and diagnosis of MBI was 29 days and was still longer for patients with atypical MBI or recipients of corticosteroids therapy. Recently, an ex vivo enzyme-linked immunospot (ELISPOT) assay for interferon-γ (IFN-γ) produced by T cells specific for two gene products of Mycobacterium tuberculosis (MT): the early secretory antigen target-6 (ESAT-6) and culture filtrate protein 10 (CFP-10), has been demonstrated as a rapid, highly sensitive and specific technique for the detection of MT infection both in immunocompetent and Human Immunodeficiency Virus infected patients with active, culture-confirmed and or with latent TBC. Here we report the case of an HCL patient presented with persistent fever, bilateral pleural effusions and multiple hypointense splenic lesions. No common infections were documented. Tuberculin skin test resulted negative. Wide-spectrum antibiotics were ineffective. An ex-vivo ELISPOT assay detected, both on peripheral blood and pleural fluid samples, high level of interferon-γ, produced by T-cells reactive versus ESAT-6. On the basis of the ELISPOT assay, although in the absence of other confirmatory results, the patient underwent a four drugs anti-tubercular (TBC) treatment with resolution of the clinical syndrome. Only three weeks later the beginning of anti-TBC therapy, the blood culture taken at admission became positive, confirming the ELISPOT diagnosis of disseminated MBI. We provide the first clinical application of ELISPOT as new tool for the early and specific diagnosis of MBI in hematologic patients. Ex vivo ELISPOT may be potentially useful in term of clinical management and cost-effectiveness. Further studies on large number of patients are warranted to validate the clinical efficacy of ELISPOT in hematologic patients.

**PO-184 GLUTATHIONE S-TRANSFERASE (GST) P1 GENOTYPE AND PROGNOSIS IN HODGKIN’S LYMPHOMA**

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Glutathione-S-Transf erase (GST) P1 is a member of the GST enzyme superfamily which is important for the detoxification of several cytotoxic drugs and their by-products. A single nucleotide polymorphism results in the substitution of Isoleucine (Ile) to valine (Val) at codon 105 causing a metabolically less active variant of the enzyme. Recently, the GSTP1 105Val genotype has been associated with a favorable prognosis following chemotherapy with drugs known to be GSTP1 substrates in a variety of malignancies, such as pediatric acute lymphoblastic leukemia, myeloma, breast and colon cancer. We assessed the impact of the GSTP1 codon 105 genotype, as well as deletions of GSTT1 and GSTM1, and polymorphisms of CYP1A1 on treatment outcome in 97 patients with Hodgkin’s lymphoma. Eleven patients (11%) were homozygous for the 105Val/105Val genotype, 36 (37%) were heterozygous (105Ile/105Val) and 50 (52%) were homozygous for 105Ile/105Ile GSTP1 genotype. Our observed allele frequency for the GSTP1 105Val allele was 0.30 (58/194) and was similar to previous reports on allele frequencies for healthy Caucasians. The GSTP1 Ile105Val polymorphism was associated in a dose-dependent fashion with an improved failure-free survival in patients with Hodgkin’s lymphoma (p=0.02). The probability of 5-year survival for patients homozygous for the 105Val/105Val GSTP1 genotype was 100%, for heterozygous patients 74% (95% CI, 56-85) and for patients homozygous for the 105Ile/105Ile genotype 43% (95% CI, 23-61). When the analysis was restricted to 53 patients treated with ABVD chemotherapy, the essentially same differences in failure-free survival were observed (p=0.02) The Cox multivariate analysis showed that GSTP1 codon 105 genotype was an independent prognostic factor. There was no association with clinical and pathologic characteristics including age, sex, histotype, stage of disease, presence of B-symptoms, bulky disease and abnormal laboratory parameters. In conclusion, our results indicate a strong prognostic impact of the GSTP1 genotype on the outcome of patients with Hodgkin’s lymphoma treated with chemotherapy.
Hepatitis C Virus (HCV) is largely, although not homogeneously, diffuse in several countries of the world. It has been shown to play a role both in hepatocellular carcinoma and in B-cell non-Hodgkin lymphoma (B-NHL). Up to now the exact biological mechanisms that could explain the lymphomagenic role of the virus are unknown, although several hypotheses are under investigation. In this study the role of antiviral (anti HCV) treatment in B-NHL associated with HCV infection is evaluated. This multicenter experience was able to study 13 patients affected by low grade B-cell NHL characterized by an indolent course (i.e. doubling time more than 1 year, no bulky disease): two nodal marginal lymphomas, 1 follicular, 4 plasmocytoid, 4 splenic marginal, 2 extranodal marginal lymphomas. All of them underwent only antiviral treatment with pegylated interferon and ribavirin. Eight patients experienced complete or good partial haematological response that has lasted up to now with a mean follow up of 14.1±9.7 months (range 2-24 months). Only one relapse occurred after the end of treatment. Interestingly complete and good partial responses were more likely to be seen in viral genotype 2 (p=0.035) and were strictly related to the decrease of viral load under treatment (p=<0.001). Toxicity, although important, did not cause the stop of treatment in all but 2 patients, however one of them was able to achieve complete response. Time to achieve hematological response was quite long (mean 9±2, 5 months). 

**Conclusions:** This kind of experience strongly provides a role for antiviral treatment in patients affected by HCV related low grade B-cell NHL.
Background. The IPAD chemotherapy regimen - a four-drug (idarubicin, cisplatin, HD-aracytin, dexamethasone) combination scheme - was developed at our Centre as salvage therapy for patients (pts) with relapsed/refractory aggressive non-Hodgkin’s lymphomas (NHL) needing disease debulking and, in bone marrow negative pts, PBSC mobilization. Aims. To evaluate safety profile and efficacy in terms of debulking and mobilizing potential of the IPAD regimen. Methods: between March 1997 and January 2004, 39 pts (M 20, 19; median age 54 yrs, range 18-74), with relapsed/refractory aggressive NHL (29 DLCL-B including 4 primary mediastinal thymic; 5 T- large cell; 5 mantle cell NHLs) received the IPAD regimen as salvage therapy; in bone marrow negative pts, feasibility of PBSC harvest following IPAD was also assessed. A total of 80 courses were performed (median 2/pt). Response was evaluated after 2 courses of therapy using standard parameters. Results. No toxic deaths were observed. Four pts are not evaluable: 3 of 4 are too early to be evaluated, 1 died because of disease progression while receiving CT. Among the remaining 35 pts, overall response was 51.5% (18 pts, 7 CR, 11 PR); in 11 pts disease progression (P) was observed, while 6 pts had stable disease (SD) and were considered non-responders. Hematological grade IV toxicity was observed in 21 of 39 pts (54%); 4 pts (10%) developed either FUO or bacterial infections during neutropenia. PBSC harvest was performed in 12 bone marrow negative pts and it was successfull in all; among these pts median PBSC harvest was 6.5 CD34+×10^6/kg (range 2.5 - 90×10^6/kg). Eight pts underwent autologous bone marrow transplant after IPAD (2 CR, 4 PR, 1 P, 1 SD); 6 of 8 are currently in continuous complete remission (CCR) with a median follow-up of 13 months (range 1-27). Of the 5 CR pts who did not undergo transplant, 1 progressed 2 mos from CT, 2 were in CR when they were lost to follow-up after 11 and 15 mos respectively and 2 are still in CCR after 4 and 76 mos of follow-up respectively.

Conclusions. These preliminary data show that the IPAD regimen is fairly well tolerated in pts with aggressive, relapsed-refractory NHL. In our series, response was observed in approximately half of these pre-treated pts. Moreover, our data show that IPAD combination CT is a predictable and highly effective mobilization regimen in this subgroup of pts.

PO-188

POLYMORPHISMS OF THE INTERLEUKIN 10 GENE PROMOTOR AND SUSCEPTIBILITY TO INDOLENT NON-HODGKIN LYMPHOMAS


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Epidemiologic studies on sibling of case subjects and monozygotic twins have shown the existence of a genetic susceptibility to lymphomas. However it has not yet been identified a candidate gene. Interleukin 10 (IL10) is an important immunoregulatory cytokine, mainly produced by monocytes, T cells as well as healthy and neoplastic B lymphocytes. IL10 has strong immunosuppressive effects, and it stimulates the proliferation and differentiation of B cells. Further, IL10 has been shown to have a role in the pathogenesis of lymphomas, acting as an autocrine growth factor which up-regulates bcl-2. In vitro studies have shown that specific polymorphisms located in the promoter of the gene are associated with different levels of IL10 production. In particular it has clearly been shown that the single nucleotide polymorphism in position 1082 (IL10-1082) has a notable influence on IL10 production. Likewise, specific Epidemiologic studies on sibling of case subjects and monozygotic twins have shown the existence of a genetic susceptibility to lymphomas. However it has not yet been identified a candidate gene. Interleukin 10 (IL10) is an important immunoregulatory cytokine, mainly produced by monocytes, T cells as well as healthy and neoplastic B lymphocytes. IL10 has strong immunosuppressive effects, and it stimulates the proliferation and differentiation of B cells. Further, IL10 has been shown to have a role in the pathogenesis of lymphomas, acting as an autocrine growth factor which up-regulates bcl-2. In vitro studies have shown that specific polymorphisms located in the promoter of the gene are associated with different levels of IL10 production. In particular it has clearly been shown that the single nucleotide polymorphism in position 1082 (IL10-1082) has a notable influence on IL10 production. Likewise, specific alleles in the tandem repeated region at locus 1064 (IL10-1064) have been demonstrated as important factors for graft-versus-host disease occurrence in the setting of allogeneic stem cell transplantation. The aim of our study was to compare the distribution of these specific IL10 polymorphisms between a population of patients affected by lymphomas.
Primary lymphoma of the breast is a rare disease, accounting for less than 2% of extranodal non-Hodgkin's lymphoma. The majority of cases are of B-cell origin and are morphologically classified as diffuse large B-cell lymphomas (DLBCL). Knowledge about the pathogenesis and histogenesis of primary breast lymphoma is scarce and new molecular markers are under study to improve the characterization of the disease. Molecular markers of histogenesis are represented by somatic mutations of IgV and BCL6 genes, that are physiologically acquired by B-cells during T-cell dependent antigen reaction and characterize lymphomas derived from germinal centre (GC) or post-GC B-cells. The physiological mechanism of somatic hypermutation may be malfunctioning in lymphoma, causing the accumulation of somatic mutations of PAX-5, RhoH/TTF, PIM-1 and c-MYC proto-oncogenes, a process known as aberrant somatic hypermutation.

In this study, we aimed to clarify the molecular histogenesis of primary breast lymphomas by defining the mutational status of IgV and BCL6 genes. Also, we aimed at understanding the pathogenetic role of the aberrant somatic hypermutation process in primary breast lymphoma by analyzing the mutational status of PAX-5, RhoH/TTF, PIM-1 and c-MYC proto-oncogenes. Toward this aim, 13 cases of primary breast lymphoma were analyzed for the target genes by PCR amplification and direct sequencing. A functional IgVH rearrangement was identified in 8/13 (61.5%) primary breast lymphomas. All rearrangements of IgVH genes detected in primary breast lymphoma displayed somatic hypermutation, with a mutation rate ranging from 4.0% to 25.9%. These data suggest a derivation of primary breast lymphomas from GC or post-GC B-cells. The IgVH gene families utilized included VH4 (4/8 cases), VH3 (3/8 cases) and VH2 (1/8 case). Mutations of BCL6 were detected in 7/13 (53.8%) cases, further confirming the finding that primary lymphomas of the breast derive from GC-experienced B cells. Analysis of aberrant somatic hypermutation of proto-oncogenes was performed on selected regions known to contain >90% of mutations found in lymphoma. Overall, mutations in at least one of the four proto-oncogenes targeted by aberrant somatic hypermutation were found in 9/13 (69%) primary breast lymphomas, whereas mutations in more than one gene were found in 4/9 (44%) cases. Each of the four proto-oncogenes was altered in a significant fraction of primary breast lymphomas (PAX-5 in 4/9 cases; RhoH/TTF in 5/9 cases; PIM-1 in 5/9 cases and c-MYC in 2/9 cases). The overwhelming majority of mutations was represented by single base-pair substitutions (n=36), whereas in only one instance a deletion of a short DNA stretch was observed. Among the 36 single base-pair substitutions detected in primary breast lymphoma, the transition/transversion ratio was 1.76 (expected 0.5; \( p<0.01; \chi^2 \) test), reflecting the mutational profile seen in nodal DLBCL of immunocompetent hosts. In PIM-1, a fraction of mutations led to amino acid substitution, with potential functional consequences. In particular, three primary breast lymphomas displayed four missense mutations localized within the serine-threonine kinase domain of PIM-
The association of primary breast lymphoma with aberrant somatic hypermutation of proto-oncogenes expands the types of aggressive lymphomas marked by this molecular abnormality and provides clues for understanding breast lymphoma pathogenesis. In particular, missense mutations in the PIM-1 coding region can deregulate its function, whereas mutations of the 5’ regulatory regions of PAX-5, RhoH/TTF and c-MYC are expected to influence the expression and regulation of these genes in a fashion similar to that reported for the BCL-6 gene in B-cell lymphoma. Consistent with the role of PAX-5 in B-cell differentiation, of RhoH/TTF in signal transduction, and of c-MYC in B-cell growth and fate, deregulation of these genes by aberrant hypermutation may contribute to breast lymphoma pathogenesis by multiple pathways.

PO-190
HHV-8 POSITIVE PEL, LONGSTANDING KAPOSI SARCOMA AND IDIOPATHIC CD4+ T-LYMPHOCYTOPENIA

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Human herpesvirus (HHV-8) associated primary effusion lymphoma (PEL) occurs more frequently in the context of HIV-infection. We describe the case of a HHV-8-positive PEL in a HIV-negative Italian woman with idiopathic CD4+ T-lymphocytopenia. At the age of 36 years, the patient developed recurrent effusions in the pleural cavity after longstanding (> 10 years) Kaposi sarcoma (KS). Serologic test for HIV, HBsAg and HCV were negative. PCR analysis of PBMCs resulted positive for HHV-8 sequences 7 years before the occurrence of PEL. For several years the patient had low number of total WBCs, CD3+, CD4+, and CD8+ T cells. At the same time, she had hepatosplenomegaly and lymphadenopathies that were never biopsied. We examined 6 effusion samples in the course of her last 6 months of life. These effusions contained several cell types: macrophages, lymphocytes, eosinophils, neutrophils, mesothelial cells, and a small proportion of atypical lymphoid cells of medium-large size together with apoptotic bodies. Molecular studies of the pleural fluid sediment disclosed HHV-8 but not EBV DNA, and no clonal rearrangements of IgVH, IgVL and TCR genes. HHV-8 was detected in saliva and again in PBMCs. The cytokine profile of pleural fluids is in progress. This case is an unusual example of PEL for two reasons. First, it occurred in the context of a severe form of immunodeficiency unrelated to HIV-infection that likely favoured the activation of HHV-8. Second, it occurred in a woman; only few papers have described PEL in women, including 6 HIV-negative cases (Said et al. Blood 1996; Carbone et al. Br J Hematol 1996; Codish et al. Am J Hematol 2000; Nitsu et al. Ann Hematol 2000; Boulanger et al. Am J Hematol 2004) and 1 HIV-positive case (Valencia et al. AIDS 1999). Another peculiarity is that the patient originated from a geographical area in Campania characterized by high incidence of classic KS and not negligible rates of HHV-8 infection (Montella et al. J Viral Hepatol 2004).

PO-191
MINIMAL RESIDUAL DISEASE HAS NOT A SIGNIFICANT IMPACT ON LONG-TERM OUTCOME OF PATIENTS WITH AGGRESSIVE LYMPHOMA

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Various molecular markers, ie IgH, Bcl2, Bcl1 and TCR rearrangements, are now available for both molecular diagnosis and for the evaluation of minimal residual disease (MRD) in patients with non-Hodgkin’s lymphomas (NHL). Although these molecular assays are sufficiently sensitive and specific, their predictive role in minimal residual disease detection is still controversial. In a series of 211 patients affected by aggressive NHL, autologous transplantation offered a longer PFS only in high-risk cases, where 5-year PFS was 66% for transplanted patients vs 44% for cases treated by conventional chemotherapy. Fifty-eight of these patients (88% with diffuse large cell B LNH) underwent autologous transplantation (PBSCT) and were evaluated for the presence of molecular markers (IgH and Bcl2/JH rearrangements) on bone marrow (at diagnosis and after graft) and on harvests in order to find a possible predictive role of MRD on treatment outcome. At diagnosis, 37% of patients showed molecular involvement of bone marrow samples; 33% of these cases have been negative at the histological evaluation. The PCR-positive was significantly correlated with advanced stage of disease. Thirty-two percent of leukaphereses resulted PCR-positive, mainly when patients were mobilized in advanced stage of disease or showed a molecular involvement of bone marrow at diagnosis. Interestingly, 13% of patients with PCR-negative bone marrow at diagnosis harvested PCR-positive precursors. Thirty-six
percent of aphereses underwent ex vivo purging, but only 3% became IgH-negative. Five-year OS was 90% and PFS 71%; molecular status of aphereses, included the ex vivo purging, did not significantly condition long-term prognosis of our patients. With a median follow-up of 37 months, after PBSCT 90% of tested patients were PCR-negative; the relapse rate was not different between MRD-positive and negative cases. In conclusion, in opposition to that observed in indolent lymphomas, serial molecular monitoring of MRD using qualitative PCR techniques could not represent a real prognostic indicator for patients affected by aggressive NHL.

**PO-192**
**USE OF A REAL-TIME-PCR ASSAY TO IMPLEMENT STAGING PROCEDURES IN PATIENTS WITH T(14;18) POSITIVE FOLLICULAR CELL LYMPHOMA**


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Follicular Cell Lymphoma (FCL) is one of the most frequent adult non Hodgkin’s lymphomas (NHL) and its incidence increases with age. Generally it presents an indolent clinical course but is not curable and may progress to diffuse large B cell lymphoma. At diagnosis, a diffuse lymphadenopathy and a disseminated disease (stage IV) are frequent. FCL originates from germinal center B-cell carrying the t(14;18) translocation which results in the juxtaposition of the BCL-2 gene to the joining region (JH) of immunoglobulin heavy chain. The BCL-2/JH rearrangement can be easily detected by qualitative PCR assay, in about 60-80% of FCL patients, on DNA samples obtained by lymph nodes (LN), bone marrow (BM) or peripheral blood (PB). t(14;18) detection by conventional PCR assay provides a useful tool for i) diagnosis, ii) monitoring of minimal residual disease (MRD), iii) early evaluation of the efficacy of new therapies (e. g. Rituximab) and iv) gaining relevant prognostic information in FCL. The use of very sensitive nested-PCR assays, however, may result in the detection of BCL-2 rearrangement in PB of about 25% of normal individuals (frequency increasing with age). The development of a quantitative PCR strategy, theoretically able to find a cut-off value between the amounts of positive clones found in FCL patients versus those found in healthy subjects, may overcome such potential pitfall. We developed a REAL-Time PCR strategy able to quantify the number of cells carrying the BCL-2/JH rearrangement by the taqman technology. The absolute quantification was obtained by serial dilution of a cloned primer-specific template, exactly quantified by competitive PCR against a competitor designed by selecting a sequence not present in the human genome to which BCL-2 specific primers were linked. The sensitivity of our REAL-Time PCR assay was assessed by a serial dilution of DNA extracted from DoHH2 cell lines (carrying BCL-2/JH rearrangement) and expressed as number of alleles in 500 ng of DNA (75000 cells). In our hands the limit sensitivity of the assay was 40 cells/500 ng DNA (5.3×10^{-4}). We then exploited the assay to implement staging procedure at diagnosis in a cohort of 28 FCL patients undergoing chemotherapy with fludarabine + rituximab containing regimens. The absolute amount of t(14;18) positive cells was comparatively assessed in LN, BM and PB samples at diagnosis. The mean number of alleles detected in 500 ng of DNA (75000 cells) from BM and PB was very similar in our cohort of patients (104.8 and 84.1 respectively), while LN samples yielded a significantly higher mean value (10712.3). Real-time PCR results were correlated with disease parameters at diagnosis, including clinical stage, bulky disease, bone marrow involvement, IPI score, performance status, extra-nodal site involvement and presence of B symptoms. Interestingly, a positive correlation emerged between the number of positive cells detected at diagnosis on lymph nodes, but not in PB, and an increased risk of BM and extra-nodal sites involvement. These results, if confirmed on a larger number of patients, may constitute the basis for a molecular staging in FCL, provide a new molecular tool to assess the risk of disseminated disease at diagnosis and a new important prognostic factor to orientate the therapeutic strategy.

**PO-193**
**ANGIOIMMUNOBLASTIC T-CELL LYMPHOMA AND EBV-ASSOCIATED LARGE B-CELL LYMPHOMA : A CASE REPORT**


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Angioimmunoblastic T-cell lymphoma (AITL) is a systemic disease characterized by the monoclonal proliferation of T cells expressig CD3 and CD4. In most patients, the tumor cells are greatly undervaluated by numerous relative cells. This raises some difficulties, regarding both diagnosis and the investigation of the biological characteristic of the tumor cells. The clinical outcome of these patients remains bleak, despite improvements in the management of other aggressive lymphomas. We report a rare case of angioimmunoblastic T-cell lymphoma (AITL) and diffuse large B-cell lymphoma occurring in a 56-year-old woman
with generalized lymphoadenopathy and hepatosplenomegaly. The patient initially was admitted at a local hospital, in May 2003, because of fever, sweats, fatigue, generalized pruritus, pharyngodinia and with a single left cervical lymphadenopathy. Histologic examination of the cervical lymphnode was interpreted as atypical immunoblastic proliferation. After treatment the clinical symptoms disappeared and laboratory findings normalized. She developed generalized lymphadenopathy 10 months later and was referred to our institution for further evaluation. Laboratory analysis showed dysproteidemia with polyclonal hypergammaglobulinemia, leukocytosis with eosinophilia, anemia and thrombocytopenia. The patient presented also a pneumoniae pulmonary infection (chest X-ray showed a ground glass aspect) and a EBV infection (identified by the polymerase chain reaction). The recent biopsy of the cervical node showed typical features of AILT. Flow cytometric immunophenotyping identified an aberrant CD4+ T-cell population that lacked surface CD3. Polymerase chain reaction analysis of the T-cell receptor \( \gamma \) gene revealed a clonal rearrangement. In addition to the AITL, the lymph node showed partial involvement by a diffuse large B-cell lymphoma. The B lymphoma cells and the admixed immunoblasts and Reed–Sternberg-like B cells were positive for Epstein–Barr virus (EBV) by \( \textit{in situ} \) hybridization. Our findings raise the possibility that EBV-associated large B-cell lymphoma is a secondary event in AITL via EBV infection or reactivation followed by clonal expansion of an immortalized EBV-infected B cell clone. The future success in dealing with AITL will depend on the progress in understanding the biology of the disease and in establishing international collaborations to test biological discoveries in large clinical trials.

PO-194
IMMUNOPHENOTYPIC PROFILE AND ROLE OF ADHESION MOLECULES IN SPLENIC MARGINAL ZONE LYMPHOMA WITH BONE MARROW INVOLVEMENT
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Splenic marginal zone lymphoma (SMZL), with or without villous lymphocytes, is listed as a well-defined entity in the World Health Organization classification of lymphoid tumours. The spleen is reported as being the primary site of involvement. In bone marrow, which is invariably involved, different patterns of infiltration have been described: intrasinusoidal, interstitial, nodular, para–trabecular, and packed. Adhesion molecules constitute a heterogeneous group of antigenic receptors which play a major role in leukocyte rolling, adhesion and extravasation, in lymphocyte homing and in various phases of cellular–mediated immune response. It has been supposed that a variable expression of adhesion molecules on SMZL lymphocytes may influence the evolution and pattern of the BM infiltrate. This study was performed on 38 patients (18 males; 20 females; median age, 66 years; range, 23–79), with diagnosis of SMZL (21 patients) or of Splenic Lymphoma with Villous Lymphocytes (17 patients). All patients underwent a bone marrow biopsy at the time of the diagnosis in order to evaluate bone marrow involvement. Histological (haematoxylin and eosin, periodic acid Schiff, Giemsa, and Gomori) and immunophenotypical (Psgl-1 (CD162), E-selectin (CD62-E), L-selectin (CD62-L), HCAM (CD44), ICAM-1 (CD54), and \( \beta-1 \) integrin (CD29)) stainings were performed in order to analyse the frequency, degree and pattern of bone marrow infiltration and evaluate the expression of a set of adhesion molecules among the different patterns of infiltration. A small–B-cell, mature appearing, lymphoid infiltrate was detected in all patients. The intrasinusoidal pattern of infiltration was constantly observed, alone or in conjunction with other patterns, most frequently nodular or interstitial. As regards adhesion molecule immunophenotype, PsGL1 expression was limited to a low percentage of neoplastic lymphocytes, mainly in the perisinusoidal region (Figure 1) and in the peripheral zone of the nodules (Figure 2).

Figure 1. ICAM-1 was selectively expressed in the core region of the nodules (Figure 3) while the intrasinusoidal and interstitial infiltrates were negative, and HCAM was constantly positive in most of neoplastic cells independently from the infiltration pattern. E-selectin, L-selectin and \( \beta-1 \) integrin were constantly negative. These data strengthen the hypothesis that bone marrow pattern of infiltration in SMZL/SVL is influenced by adhesion molecule expression.
PO-195
INTRAVENOUS ADMINISTRATION OF RITUXIMAB IN CENTRAL NERVOUS SYSTEM LYMPHOMAS
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Introduction: Anti-CD20 Monoclonal antibody Rituximab is a pivotal compound for the treatment of the majority of CD20+ non-Hodgkin lymphomas. Central nervous system (CNS) lymphomas are infrequent lymphomas, generally of the diffuse large cell lymphoma (DLCL) type, that localise either in the leptomeningeal compartment or in the parenchimal compartment (or both) of the CNS. CNS lymphomas have a bad prognosis and current treatments are all unsatisfactory. We investigated the distribution and efficacy of intravenous rituximab in patients with systemic DLCL with documented CNS involvement, refractory to conventional treatments.

Methods: Rituximab was administered intravenously at 375 mg/m² in 3 patients with CNS DLCL. Prior to rituximab infusion, Lumbar puncture had demonstrated leptomeningeal involvement with neoplastic cells in the cerebro-spinal fluid (CSF) in 1 case, while sole parenchimal involvement had been demonstrated in the remaining 2 cases by MRI of the CNS. Serum and liquoral antibody levels were measured concomitantly before and after intravenous administration of rituximab (375 mg/m²) at different time points. Concentration of rituximab was measured using an inhibition assay in flow cytometry, by incubating CD20+ Raji cell line and PE conjugated anti-CD20 MoAb (Pharmingen) in the presence of serum or CSF.

Results: Rituximab was documented in the CSF of all 3 patients, with a maximum concentration that was reached within the first 24-48 hours from the time of infusion. Plateau concentrations were maintained for more than a week after infusion and remained detectable even after 2 weeks from infusion. In 2 patients where serum and CSF concentrations could be compared, we observed that from 1 to 10% rituximab passed from the serum to the CSF. In the 2 cases with parenchimal involvement, subsequent clinical and/or MRI evaluation of the parenchimal masses documented scarce benefit on the CNS involvement. Interestingly, however, the patient with leptomeningeal involvement demonstrated a rapid clearance of tumor cells from CSF.

Conclusion: our preliminary data demonstrate that rituximab administered to patients with CNS lymphoma can pass the blood-brain barrier and that it can reach concentrations with potential therapeutic activity, which can be effective in patients with leptomeningeal involvement.
PO-196
EPRATUZUMAB/SAPORIN-S6: A NEW ANTI-CD22 IMMUNOTOXIN WITH A POTENT ANTITUMOUR ACTIVITY
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The use of monoclonal antibodies as anti-cancer therapies has been widely explored since their initial development by Kohler and Milstein. In contrast to chemotherapy and radiotherapy specific antibodies can preferentially bind tumor cells over normal tissues. The vascular nature of most lymphomas suggests that they may represent a favorable setting for this treatment modality. In fact, the first successful use of antibodies as treatment for cancer was demonstrated in lymphoma, and these agents have now been employed to benefit thousands of patients with non-Hodgkin’s lymphomas (NHL). CD22 is expressed at high levels on normal mature B-cells and on a large proportion of B lymphoma cells. The humanized anti-CD22 antibody Epratuzumab (hLL2) was used in clinical trials giving good anti-tumor activity. It was well tolerated, 16% of the patients responded and one third of them achieved CR in a Phase I/II trial. Better results could be obtained coupling this mAb to a toxin to obtain a chimeric protein, defined immunotoxin. In our studies we linked Epratuzumab to the RIP saporin–S6. RIPS are plant toxins with RNA N-glycosidase activity, which cleave one or more adenine molecules from ribosomal RNA, thus damaging ribosome in an irreversible manner. The effects of Epratuzumab-saporin–S6 were evaluated as inhibition of protein synthesis on five target B-lymphoma cell lines: BJAB, REH, D430B, Raji and Ramos. Time-course and dose-response experiments were performed to extrapolate the kinetics of protein synthesis inhibition and the concentration of immunotoxin giving 50% inhibition (IC50). Conjugation with Epratuzumab enhanced saporin–S6 cytotoxicity on target cells by at least 3 logs, with IC50 in the pM range. No protein synthesis inhibition was induced by free mAb. Protein synthesis of CD22-negative Jurkat cells was not affected by the immunotoxin at concentrations up to 10 nM. These results were confirmed by another cytotoxicity assay, based on MTS. We further demonstrated the killing efficiency of the immunotoxin using a very sensitive luminometric method, measuring the ATP produced by surviving cells, in time- and dose-response experiments. Moreover, as the final target of an anti-tumour therapy is to completely eradicate the disease, the clonogenic growth of target cell lines was determined after exposure to anti-CD22 immunotoxin. A complete elimination of BJAB clones was reached after a short time (3 h) exposure to the conjugate, at 10 nM concentration vs a 15% inhibition of clonogenic growth reached with saporin–S6 alone.

References
PCR analysis of the pleural sediment disclosed no revealed nuclear staining in atypical lymphoid cells. Bodies to the latent nuclear antigen (LNA-1) ORF 73 clear cells, saliva and in the pleural sediment. Anti-were detected by PCR in peripheral blood mononuclear samples. HHV-8 DNA sequences, but not EBV, the first fluid but increased in number in the subsequent samples. Atypical lymphoid cells that were not numerous in occurred in November. Effusions contained large recurrent pleural effusions, high fever and widespread viral therapy. Nonetheless, the patient developed lymphoadenopatic, visceral and mucocutaneous KS. The course of disease was aggressive and death in Italy.

Human herpesvirus 8 (HHV-8) associated primary effusion lymphoma (PEL) and Kaposi sarcoma (KS) are diseases primarily affecting men. HHV-8 is highly prevalent in many African countries, wherein it is as common in women as in men. Since the epidemic of HIV, KS has become relatively more frequent in women and it can be aggressive and devastating (Nnoruka et al. Int J Dermatol 2003). We herein report the case of a HHV-8-positive PEL in a HIV-positive 26-year-old black woman. She was born and lived for 22 years in Cameroon, central Africa, then 4 years in Italy. The patient was graduated student who denied intravenous drug abuse and declared heterosexual unprotected intercourse. Her past medical history was negative except for malaria during childhood/adolescence. In July 2003 she became symptomatic with progressive malaise. In August she developed a febrile syndrome associated with Shigella flexineri, and received a blood transfusion because of severe anemia (Hb 7.0 g/dL). Serologic test for HIV was positive (HIV-RNA 5300 copies); CD4 61%. She underwent highly anti-retroviral therapy. Nonetheless, the patient developed recurrent pleural effusions, high fever and widespread lymphoadenopatic, visceral and mucocutaneous KS. The course of disease was aggressive and death occurred in November. Effusions contained large atypical lymphoid cells that were not numerous in the first fluid but increased in number in the subsequent samples. HHV-8 DNA sequences, but not EBV, were detected by PCR in peripheral blood mononuclear cells, saliva and in the pleural sediment. Antibodies to the latent nuclear antigen (LNA-1) ORF 73 revealed nuclear staining in atypical lymphoid cells. PCR analysis of the pleural sediment disclosed no clonal rearrangements of the Ig heavy chain gene. The failure of Ig PCR in PEL has been reported previously (Fais et al. Leukemia 1999; Hamoudi et al. Leukemia Research 2004). Sequencing of the ORF K1 gene is in progress to document the HHV-8 strain. This case is an example of HHV-8 associated PEL in an African woman with HIV-related immunodeficiency. Few papers have described PEL in women: 6 HIV-negative cases (Said et al. Blood 1996; Carbone et al. Br J Hematol 1996; Codish et al. Am J Hematol 2000; Niitsu et al. Ann Hematol 2000; Boulanger et al. Am J Hematol 2004) and 1 HIV-positive case in which, however, the proof of HHV-8 infection is lacking (Valencia et al. AIDS 1999). To our knowledge, PEL has never been reported in Africans. In Cameroon, HHV-8 seroprevalence is high (28–62%; up to 55% in pregnant women), HHV-8 infection takes place during childhood by non-sexual casual routes, and KS was relatively frequent also before the spread of HIV infection epidemic (8/1000 men) (Gessain et al. Int J Cancer 1999).

Anthracyclines are widely used in the treatment of lymphomas but their clinical efficacy can be limited by acute and chronic toxicity, in particular cumulative cardiac damage. Several approaches have been attempted to reduce cytotoxicity of these antineoplastic agents, such as the use of drugs with potential cardio-protective action. Dextrazoxane clorhydrate (Cardioxane®), a synthetic bisdiketopiperazine two-ringed compound which hydrolyzes to an EDTA analog, seems to be able to reduce cardiac toxicity by binding to free and bound iron, thus reducing the formation of anthracycline-iron complexes and the generation of free radicals which are toxic to cardiac tissue. In daily practice, parameters of systolic function (left ventricular ejection fraction or fractional shortening) are employed to detect cardiotoxicity, but these methods are not able to identify acute cardiac damage. The determination of QT dispersion may identify patients at risk of the development of early heart failure. The main purpose of the present study was to assess the effect of epirubicin-based chemotherapy (PROMECECytaBOM) on plasma free radical
PO-199
POOR MOBILIZATION IN DIFFUSE LARGE CELL LYMPHOMAS: PREDICTIVE FACTORS AND IMPACT ON SURVIVAL

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Haemopoietic stem cell therapy is an increasing procedure in the treatment of patients (pts) with diffuse large cell lymphoma (DLCL). A good mobilization is mainly achieved by combining chemotherapy and Haemopoietic growth factors. Nevertheless a 10–20% of pts with malignant lymphomas doesn’t reach the minimum threshold of 2x10^6 CD34+ collected cells/kg and are considered as poor mobilizer pts. In our retrospective analysis, we evaluated 143 pts with DLCL in order to evaluate the impact of the poor mobilization on pts survival determined as OS and EFS and to establish the factors possibly predictive of a CD34+ poor mobilization. Mobilization chemotherapy regimens were: high dose cyclophosphamide in 38 (26.5%), high dose cytarabine containing regimen (DHAP in 51 pts -35.7%, MAD in 54 -37.8%). All pts received subcutaneous lenogastrim from day +2 after chemotherapy. Univariate and multivariate statistical analysis concerning some hematologica parameters (WBC, HB, PLT, CD34+ cells) at diagnosis, at the moment of the mobilization regimen and at harvest and clinical parameters (age, histology, disease status, BM involvement at diagnosis and at mobilization, chemotherapeutic load) were evaluated. The incidence of poor mobilizer pts (CD34+ cells ≤ 2x10^6/kg) was 14.6% overall with a 8% of very poor mobilizers (≤ 1x10^6/kg). Refractory disease status and previous chemotherapeutic load before mobilization play a negative role to determine a poor mobilization effect. The Survival analysis of all harvested pts showed an Overall Survival (OS) at 3 years of 22% in poor mobilizer group vs. 59% in good mobilizer (p=0.0014); the Event Free Survival (EFS) was 12% in poor mobilizer and 59% in good mobilizer (p=0.0007). In DLCL, poor mobilization status is a statistical significant predictive factor of a waste survival (OS and EFS). Further prospective studies, to establish the best mobilizing procedure and a possible alternative mobilizing procedure, are warranted.

The patients were randomly allocated to receive or not dexrazoxane clorhydrate (40 mg/m²) after epirubicin infusion. Peripheral blood samples in EDTA were recovered at baseline, after epirubicin infusion, and one hour later. Oxidative stress was evaluated by measuring at the beginning and at the end of the treatment plasma malondialdehyde (MDA) and plasma antioxidant capacity as its ability to antagonize the oxidation of α-keto-gamma-methylbutyric acid by hydroxyl radicals. The results are expressed as total oxyradical scavenging capacity (TOSC) (1.9±5.1 vs. 27.4±6.8 U, p<0.001). This increase is not found in placebo-treated patients (12.8±3.7 vs. 18.5±4.2 U, n. s.). Moreover, all participants underwent 12-lead electrocardiogram (ECG) at baseline, after epirubicin infusion, and one hour later. QT intervals were measured from surface electrocardiograms and QT dispersion was defined as maximum QT - minimum QT occurring in any of the 12 leads. QT dispersion was corrected (QTc) for heart rate according with Bazett’s formula.

Results. One hour after the end of epirubicin infusion, plasma MDA was significantly increased (2.8±0.3 vs. 5.8±1.1 mol/l, p<0.001) and plasma TOSC against hydroxyl radicals significantly reduced (33.2±8.4 vs. 12.5±4.3 U, p<0.0001) in the whole study population. After epirubicin infusion, dexrazoxane clorhydrate induced a significant increase of anti-hydroxyl radicals antioxidant capacity (11.9±5.1 vs. 27.4±6.8 U, p<0.001). This increase is not found in placebo-treated patients (12.8±3.7 vs. 18.5±4.2 U, n. s.). Moreover, all patients showed increased QT dispersion (44.3±8.4 vs. 68.4±11.4 ms, p<0.001) and QTc dispersion (46.2±6.2 vs. 72.2±8.4, p<0.001) during epirubicin therapy. Interestingly, patients who were supported with dexrazoxane clorhydrate exhibited a significant reduction of both QT (67.4±8.1 vs. 49.5±4.2 ms, p<0.001) and QTc dispersion (71.2±7.7 vs. 51.4±4.3 ms, p<0.001), if compared to placebo group (QT 69.3±7.6 vs. 64.2±6.9 ms; QTc 72.8±8.1 vs. 67.3±7.2 ms, ns). Conclusion. These results show a significant dexrazoxane activity against anthracyclines-induced damage to myocytes by preventing iron-based oxygen radical formation. Furthermore, it has been showed that anthracycline-based chemotherapy is associated with an increased QT interval dispersion supporting the role of QT dispersion as a sensitive tool to identify the first signs of cardiotoxicity induced by anthracyclines. Dexrazoxane was able to attenuate QT interval dispersion.
Main obstacle for development of cancer vaccines is that tumor antigens are often non-immunogenic and vaccines do not elicit antigen specific cellular responses. The delivery of target antigens in a DNA vaccine format allows to include molecules with immunological properties that can improve the efficacy of the immunization. Recently we demonstrated that tumor-derived Idiotype fragments (scFv) genetically fused with proinflammatory chemokine-Id antigenic fusion proteins facilitated the uptake and presentation of antigens via the MHC class II pathway, is the mechanism by which chemokine fusion constructs elicit responses, in vitro and in vivo. Experiments with inhibitors of intracellular trafficking suggest that chemokine-antioxidant fusion proteins were processed and presented through early/late endosomal and Golgi compartments, and stimulated significant IFN-gamma release by an antigen-specific CD4+ T cell clone. This observation was also confirmed using human fusion proteins; specifically, chemokine fusion proteins facilitated the uptake and presentation of a human lymphoma Idiotype fragment (sFv), and stimulated a patient derived tumor-specific CD4+ T cell line. Next, we wanted to further increase efficacy of the chemokine-based vaccines by combining them with enhanced immunogenicity of large aggregates, such as a self-assembled 24-nm HBsAg particles, a source of current human HBV vaccine. We generated vaccine formulations expressing chemokine-Id on the surface of HBsAg and tested in mice whether these chimera would elicit anti-Id responses. Our preliminary results demonstrate that recombinant HBsAg, carrying the chemokine Id-fusions, elicits both anti-HBsAg and Id-specific antibodies. The strategy we describe is simple and potent and in combination with DNA vaccination strategy may be used for immunotherapy of B cell malignancies and other clinically relevant diseases.
CD16/CD56+ (NK) cells and CD8/CD57+ (CTL) cells in the BM (10% and 2%, respectively) and PB. Based on these results, the patient was started on high-dose steroids, methotrexate and vincristine, associated to supportive therapy with blood transfusions and growth factors (erithropoietin plus G-CSF), without improvement of the pancytopenia, which persisted for the subsequent 40 days. The patient was therefore switched to Cyclosporin-A (CSA; 400 mg/day) and after three weeks the hemogram started to steadily improve reaching acceptable values (neutrophils 1.0×10⁹/L, lymphs. 0.9×10⁹/L, Plts. 50.0×10⁹/L, Hgb 12.8 g/dL) by day +180 of continuous treatment. CSA was then discontinued and the hemogram showed a further improvement reaching normal neutrophils (3.7×10⁹/L) and Hgb (13.0 g/dL) levels by day +210. Platelet levels, however, remained as low as 60.0×10⁹/L, without further improvement to date. Immunologic studies disclosed that improvement of the hematologic picture was associated to the concurrent decrease of abnormal NK and CTL cell populations in BM and PB, which accounted for less than 1% at day +180. Molecular disease monitoring by conventional and Real-Time PCR, performed at day +210, confirmed the continuous complete remission, with absence of t(14;18)+ cells in both BM and PB. Our data indicate that CSA is effective for reversing growth factors-refractory autoimmune bone marrow aplasia triggered by Rituximab-Fludarabine combinations. Whether an immuno-mediated antineoplastic effect, exerted by NK and CTL cells, abnormally expanded after chemo-immunotherapy, might contribute to long-term disease control, remains to be established.

PO-202
DECREASED FACTOR VII ACTIVITY IN LOW-GRADE NON HODGKIN’S LYMPHOMA
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Acquired coagulation disorders associated with lymphomas are serius coagulopathy affecting elderly people with low grade lymphomas or myeloma. Usually they are due to an amyloidosis-associated decrease of factor VII and X; less frequently autoantibodies against factors VIII, IX,V are detected and rarely against factors VII, XIII and protrombin. We described a 69-year-old man with a low grade lymphoma (splenic marginal zone lymphoma ) affected by an unusual form of acquired coagulopathy. At the diagnosis all coagulative parameters were normal and a bone marrow biopsy showed a nodular localization of lymphoma. Two months after splenectomy the patient showed mild epistaxis and petechias at both legs. Routine screening tests evidenced an high international normalized ratio (INR 4.4), whereas APTT, fibrinogen,D-dimer and platelet count were normal. Clinical examination and total body TC excluded signs of progression of disease. Subsequent analysis found a normal activity of factor VIII and IX but a marked reduction of factor VII activity (40%) that was not restored by addition of fresh plasma. The patient was treated with six course of chemotherapy (cyclophosphamide vincristine prednisone ) but the INR did not change ( INR 3.9). Because of lack of major bleeding and other signs related to the lymphoma together with unchanged INR value, we stopped the therapy. After six months the patient still has an high INR, without signs of disease progression, as well as without any further bleeding. We concluded that the patient was affected by a paraneoplastic bleeding disorder probably caused by an acquired antibody directed against factor VII. We judged interesting to describe this case because the decreased factor VII activity induced only one mild epistaxis, whereas in the few published case reports factor VII antibodies are always associated to severe bleeding.

PO-203
ROLE OF OCCULT INFECTION OF HEPATITIS B VIRUS ON VIRUS REACTIVATION OCCURRING IN NON-HODGKIN LYMPHOMA PATIENTS TREATED WITH CHEMOTHERAPY
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Based on the evidence on liver tissue and blood sera of specific gene fractions of HBV (core, s and x genes) the occult infection of HBV was demonstrated. Epidemiological evidences suggest the possible role of occult infection as cofactor of disease in hepatitis C virus (HCV) related chronic hepatitis and as possible aetiological agent of liver cancer (HCC). Aim of the present study was to test the role of occult HBV infection in HBV related reactivation in patients undergone to chemotherapy for NHL. Patients and Methods: a cohort of 55 NHL patients were studied. They all were tested for routine blood examination and among all the other examinations to stage the disease, they were asked to perform liver biopsy prior to the treatment. All gave informed consent. On liver tissue and serum of all patients but HbsAg positive ones, three gene fractions of HBV were assayed (core, s and x) by PCR according to Raimondo et al.
All the patients were followed through the time of exposure to the drug and after discontinuation. Results: 18 out of 55 (33%) were HbcAB+; 15 were HCV-RNA+ (27%) and 6 HbsAg+ (10%). Prevalence of HbsAg is overestimated representing a selected group of subjects. HbsAg+ patients were treated with pre-emptive lamivudine therapy and had no HBV reactivation after chemotherapy discontinuation. Three HbcAB+ patients who were also positive for core and s genes on liver tissue experienced HBV related hepatitis reactivation. These subjects were compared to a historical group of HbsAg+ patients (n. 9) who also had a disease reactivation under the same circumstances (pre lamivudine era). In both groups lamivudine promptly dominated the pathological process and all the patients recovered. Conclusions: occult infection plays a major role in HBV reactivation in treated NHL patients as well as overt HBV infection. Nevertheless, its onset is not as frequent as in HbsAg+ patients, though the prevalence of HbcAB+ patients is high. Lamivudine pre-emptive treatment should be considered in selected class of HbcAB+ subjects.

**PO-204**

**N-GLYCOSYLATION SITES ARE INFREQUENTLY ACQUIRED IN THE B-CELL RECEPTOR OF MUTATED HAIRY CELL LEUKEMIA**

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Immunoglobulin (Ig) variable (V) region gene analysis delineates critical features of the clonal history of a B-cell tumor. It identifies whether antigen encounter by a normal mature B cell has activated somatic mutation. This is generally restricted to the germinal center (GC), and isotype switch events may also occur there. For both mechanisms, the enzyme activation-induced cytidine deaminase (AID) is critical. B-cell tumors of GC origin tend to acquire N-glycosylation sites as a consequence of somatic mutation in the VH genes. A high incidence of novel N-glycosylation sites introduced by somatic mutation is observed in the VH genes of GC derived follicular lymphoma (FL). Sites are positively selected and are uncommon in normal memory B cells, and may have a role in growth and behaviour of GC tumors. Sites are not characteristic of mutated chronic lymphocytic leukemia or myeloma, indicating no acquisition of glycosylation sites by post-GC tumors. The majority of hairy cell leukemias (HCL) have mutated VH genes, with low levels of intraclonal heterogeneity, while 15% cases carry completely unmutated VH genes (100% homology to germline). HCL commonly expresses multiple immunoglobulin (Ig) functional isotype transcripts in single cells, germline IH-CH transcripts, and AID, but not circle transcripts. These features are indicative of ongoing isotype switch events, which generally take place in the GC in normal circumstances. However, histological features and lack of GC markers in HCL, point to mutational and switching events activated by environmental factors at extrafollicular sites (Forconi F., Blood submitted). To determine whether glycosylation of the B-cell receptor is a feature of HCL, we analysed VH gene sequences of 35 HCL available from our lab (n=21) or from the literature (n=14) and scanned the deduced amino acid sequence for the introduction of N-glycosylation Asn–X–Ser/Thr (where X is any amino acid except Pro, Asp or Glu) motifs. Thirty-three out of 35 cases carried mutated VH genes (range 87-98.6%), while 2 cases carried completely unmutated VH sequences. Novel sites were rarely acquired in HCL (6/33), with a frequency (18%) comparable to that of normal memory B cells (p>0.05), and different from that of GC tumors, including FL (p<0.001) and endemic Burkitt lymphoma (p<0.001). These findings indicate that HCL does not introduce novel N-glycosylation sites. Lack of sites further supports the notion that that HCL originates from a cell that does not require interaction with GC elements to activate mutational and switching events. Absence of glycosylation sites in the VH genes of HCL provides another indicator of tumor events occurring at sites outside the GC environment.

**PO-205**

**AUTOLOGOUS STEM CELL TRANSPLANTATION IN LYMPHOMA: IMMUNOLOGICAL RECOVERY IN HIV-POSITIVE VERSUS HIV-NEGATIVE PATIENTS**

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In our Institute, refractory and/or relapsed non-Hodgkin (NHL) and Hodgkin (HD) lymphomas in HIV-positive patients (pts) are treated with HDC followed
by PBSCT, as the general population. As of December 2003, we enrolled 17 HIV-positive pts (14 M/3F; median age 40 yrs; 4 HD/13 NHL; 5 pts completed the treatment and are now in follow-up), and 10 HIV-negative pts with high grade NHL (6M/4F; median age 62 yrs; 5 completed the treatment and are now in follow-up). Before the induction therapy, mean value of CD4 count/mm(e)3 was 190+123 in HIV-positive and 340+301 in HIV-negative pts, with no significant differences. On the contrary, CD4/CD8 ratio in HIV-positive pts was significantly lower than in HIV-negative pts (0.2 vs 1.2), as well as CD56 count (54+45 vs 140+96 \( p < 0.01 \)). Before the conditioning treatment, CD4 count was still lower in HIV-positive pts, but CD4/CD8 ratio was less than 1 in both groups. CD4 count nadir was reached during aplastic period and was similar in both groups (109+104 vs 122+86). CD4/CD8 ratio was 0.2 and 0.5 in both groups, respectively. Three mos after PBSCT, CD4 count returned to baseline in HIV-positive pts (174+71), while in HIV-negative pts it was still 100 cells lower than baseline (240+95). Moreover, CD4/CD8 did not return to baseline values in HIV-negative pts and remained low as in HIV-positive pts. CD56 population returned to baseline values faster than CD4 population in both groups, but it remained significantly lower in HIV-positive pts. During follow-up no significant differences were seen in the incidence of opportunistic infections. All HIV-positive pts were on HAART and HIV-viremia was <500 copies/mL; 4 pts continued HAART during the whole treatment and their HIV-viremia remained undetectable. Our observation suggests that, despite some differences in the immunological profiles present at the beginning of the induction, no significant differences exist in the dynamic of immunoreconstitution between HIV-positive and HIV-negative pts.

**PO-206**

**2-CDA AND HIGH-DOSE CHEMOTHERAPY WITH AUTOLOGOUS PERIPHERAL BLOOD STEM CELLS IN RELAPSED LOW-GRADE NON HODGKIN LYMPHOMA PATIENTS: CLINICAL AND BIOLOGICAL FEATURES**


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Low-grade non Hodgkin’s lymphoma (NHL) remains an incurable disease when disseminated or after relapse. Therefore, new strategies have been developed including high-dose chemotherapy (HDCT) with peripheral blood stem cell rescue. Also, purine analogues as 2-CDA have demonstrated to be effective in patients (pts) with relapsed or resistant low grade NHL. Moreover, controversies still exist on the clinical relevance and the prognostic significance of the molecular marker bcl-2. We designed a sequential treatment consisting of 2 CHOP-like to reduce the tumour load followed by CTX (4-7 gram/m²) given with the purpose of both in vivo purging and mobilization of PBSC. After stem cells collection, pts received 3 cycles of 2-CDA (0.14 microgram/kilogram for 5 days every 28 days) followed by HDCT (mitoxantrone 60 milligram/square meter, melphalan 180 mg/square meter) with PBSC support. Between September 1998 and November 2002, 18 follicular NHL pts (median age 54) who relapsed after a median of 1 (1-4) regimen of chemotherapy, were enrolled. At diagnosis 8 pts were stage 2, 6 stage 3, 4 stage 4. All pts were also evaluated by PCR for bcl-2 bone marrow rearrangement before the start of the treatment: 8 pts were bcl-2 positive and 10 bcl-2 negative. Before HDCT 4 pts were in CR, 10 were in PR, 4 in SD. After HDCT, we observed 13 CR, 2 PR, 3 SD. Eight out of 10 pts bcl-2 negative before the start of the treatment and 5 out of 8 pts bcl-2 positive obtained a CR. After HDCT, median days for ANC>500/µL were 11 (8-19) and for PLT>300000/µL were 14 (3-80). Overall, 5 pts relapsed, 3 of them with bcl-2 positive and 2 with bcl-2 negative bone marrow; additional 4 pts died, 1 for PD, and 3 still in CR for severe infections. With a median follow-up of 5 years (2-12) from the diagnosis and 3 years (0.8-5.2) from the start of the protocol, 10 out of 18 pts are still in CR. These results suggest that the use of 2-CDA for NHL pts with relapsing disease is feasible and could represent a possible alternative regimen for indolent NHL pts. We didn’t observe any correlation between the bcl-2 status and the possibility to achieve a complete remission and the probability of subsequent relapse.

**PO-207**

**HODGKIN’S DISEASE IN THE BONE MARROW AND HEMATOPHAGOCYTIC SYNDROME: A CASE REPORT**


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Hemophagocytic syndrome (HPS) is characterized by systemic activation of benign macrophages showing extensive phagocytosis of hematopoietic cells. Diagnostic parameters are fever, hepatosplenomegaly, fatigue, liver dysfunction, cytopenia and hyperferritinemia, hypofibrinogenemia or hypertriglyceridemia. HPS can be associated with infections, malig-
nant lymphoma or autoimmune disease. We report a case of a 73-year-old woman with a 6-months history of weight loss, fatigue, progressive wasting and loss of appetite. She had no specific family history; deep venous thrombosis of a left lower extremity ten years before. Physical examination at admission to our hospital showed pale face, light jaundice, left submaxillary lymph node 1 cm of diameter, bilateral edema of lower extremities and few crakcles in the bilateral lower lobes. There was neither fever nor hepatosplenomegaly. Complete blood count revealed pancytopenia: white blood cell count of 1600 leukocytes per mm$^3$ with neutrophils 47%, platelets count 54000 per mm$^3$, reticulocyte count 17%; there was a mild reduction of hemoglobin level (10 g/dL). Blood chemistries were as follows: serum creatinine level 0.51 mg/dL, bilirubin 2.03 mg/dL, alkaline phosphatase 574 U/L, gammaglutamyltransferase 132U/L; serum aspartate aminotransferase 78U/L, serum alanine aminotransferase 51U/L and serum lactate dehydrogenase 751U/L. Erythrocyte sedimentation rate was 5 mm first hour, level of reactive C-protein was 0.38 mg/dL, β2 microglobulin was 7.6 mg/dL. Ferritin was 2235 ng/mL, serum gammaglobulines were very low, in particular immunoglobulines G were 326 mg/dL, immunoglobulines A 44 mg/dL and immunoglobulines M 6 mg/dL, total proteins were 3.6 g/dL and apotoglobin was 7 mg/dL. Markers for cancer (CEA, alfa fetoprotein, CA 15.3, CA 19.9) were not impaired. A severe coagulation disorder was found: prothrombin activity was 65% (INR 1.46); activated partial thromboplastin time was indeterminable; serum fibrinogen was 64 mg/dL; antithrombin III was 46%; D-Dimer 0.6 mg/L; Coombs test was normal. Eleventh and twelveth coagulation factors levels were lower than normal. Anti-nuclear antibodies (ANA) and anti-double stranded DNA antibodies (anti-ds DNA) were negative such as anti lupus coagulant antibodies (LAC). Serology including hepatitis A, B, C, HIV were negative; Chest x-ray, thoraco-abdominal computed tomography and ecocardiography were normal; abdominal ultrasound revealed a moderate hepatomegaly with diffuse hyperdense aspects like steatosis and fibrosis. During the hospitalization the general condition of patient impaired, full blood count started to deteriorate, liver function also deteriorated with light hyperbilirubinemia (bilirubin 2.9 mg/dL), hypertransaminasemia (serum aspartate aminotransferase 100 U/L and serum alanine aminotransferase 80 U/L) PT prolongation and progressive hypofibrinogenemia (48 mg/dL) without cutaneous and mucosal hemorrhagic manifestations. On the tenth hospital day the patient showed mental confusion: brain computed tomography and magnetic risonance were normal. Early cytomegalovirus (CMV) antigen expression resulted negative; nested polymerase chain reaction (PCR) assay for CMV DNA and EBV PCR on lymphocytes was positive but EBV DNA was low (twenty copies per hundred thousand cells). Serology for herpesvirus (1,2,6,8) and varicella, HBVDNA and parvovirus B19 on plasma by PCR was negative. Bone marrow aspirate showed a population of lymphocytes, macrophages, Reed–Stemnberg cells and aspects of haemophagocytosis. The multilobulated giant cells were positive for CD30 (Ki–1); NPM–ALK was negative. The diagnosis was: mixed cellularity Hodgkin’s disease associated to hemophagocytic syndrome. Combination chemiotherapy (MOPP) was started containing vincristine (1,4 mg/m², day 1,8), prednisolone (40 mg/m² day 1–14 cycles 1 and 4 only), procarbazine (100 mg/m² metre day 1–14), mechloretamine (6 mg/m² day 1,8). Coagulation test exhibited apparent recovery after MOPP but the patient quickly developed severe pancytopenia: transfusions of 3 units of packed red blood cells and 8 units of platelets were necessary. During the fourteenth chemotherapy day the patient developed fever and despite supportive therapy, antibiotic and antimicotic drugs, the clinical course was fulminant. The patient died: the cultures of blood were positive for a multidrug resistant Pseudomonas aeruginosa. The stool culture was positive for Clostridium difficile.

Conclusions. Hodgkin’s disease with involvement of the bone marrow is often associated with short survival. For this reason aggressive combination chemiotherapy is necessary. When a patient has Hodgkin’s disease associated with massive hemophagocytosis and bone marrow involvement the prognosis is bad. Aggressive chemiotherapy is the only proposed treatment but it is almost always not efficacious.

PO-208 MULTISPECTRAL IMAGING AUTOFLUORESCENCE MICROSCOPY: A NEW FIELD IN LYMPH NODE DIAGNOSIS

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Normal and pathologic cells and tissues contain molecules (fluorophores) capable of emitting fluorescent light under an ultraviolet exciting irradiation. The main fluorophores are aromatic aminoacids, flavins, lipopigments and reduced pyridine nucleotides. This phenomenon, called autofluorescence, could be studied with a Microspectrofluorometry and a Multispectral Imaging Autofluorescence Microscopy (MIAM) technique: we used this technique to analyse tissue sections obtained from lymph node biopsies performed for diagnostic purpose in patients with enlarged lymph nodes. We analyzed ten lymph...
node biopsies, three from patients with follicular reactive hyperplasia, seven from patients with neoplastic diseases (Hodgkin’s disease (four) and solid malignancy (three)); we also analysed a normal lymph node. Specimens were cryo-preserved and sections 3-4 micron thick were obtained and mounted on slides with distilled water. Cover glasses were put on the sections and MIAM analysis was carried out immediately to avoid tissue oxidation, which could cause signal changes. MIAM was performed with an apparatus constituted of an inverted epifluorescence microscope and a digital cooled camera. Monochromatic images were combined to form a single red-green-blue (RGB) colour image. Blue fluorescence emission originates from collagen, elastin and nicotinamide adenine dinucleotide phosphate; green fluorescence originates from flavins. Red fluorescence originates from lipopigments, whose tissue accumulation is due to aging and stress. Sections were also morphologically (ematoxilin and eosin stain) and immunohistochemically evaluated to compare the autofluorescence images. RESULTS: The fluorescence pattern of reactive hyperplastic nodes showed the typical lymph node organization, with low emitting lymphatic follicles separated by strongly fluorescent connective trabeculae (mainly composed by collagen and elastin). In Hodgkin’s disease the fluorescence imaging showed a loss of the normal lymph node architecture, with intense fluorescent cells often clustered in small groups. The comparison with immunostained sequential sections suggests that these highly fluorescent cells could correspond to the Reed-Sternberg’s cells. Fluorescence imaging of biopsies from patients affected from metastatic gastrointestinal carcinoma showed peculiar alterations: in a sample we observed tubular shaped structures. Autofluorescence images obtained from cryo-preserved biopsies with MIAM analysis permitted to distinguish morphological differences among neoplastic and non-neoplastic tissues, without a chemical manipulation of samples. It also offered a satisfactory comparison with standard (morphological and immunohistochemical) microscopy. This could be the background for further applications of this technique, like the development of probes for non-invasive evaluation of superficial lymph nodes and for laparoscopic and mediastinal evaluation of deep, mediastinal and abdominal lymph node.

**PO-209**

**SYNCHRONOUS DIAGNOSIS OF RELAPSING NON-HODGKIN’S LYMPHOMA AND CHRONIC MYELOMONOCYTIC LEUKEMIA IN AN OLD MAN**

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A 77-year old man was admitted on march 2002 with a history of fever, abdominal pain and vomiting. Laboratory values showed anemia (hemoglobin: 10.8 g/dl), thrombocytopenia (14×10^9/L platelets) and mild leukocytosis (14×10^9/L leukocytes: 30% neutrophils, 1% basophils, 30% lymphocytes, 49% abnormal mature monocytes). Physical examination showed liver and spleen enlargement and multiple lymphadenomegalias in all superficial sites. Bone marrow aspirate disclosed high cellularity, with monocytic maturing cells accounting for 30% of nucleated cells, three-lineage dysplastic features and lymphocyte population within normal values. The flow cytometry immunophenotyping on myeloid population was positive for CD14, CD13, CD15, CD33, CD11b, CD11c, CD4, HLA-DR and negative for CD34; no evidence of clonal excess in lymphoid population was present. Cytotypic finding and molecular analysis didn’t show abnormalities. Bone marrow biopsy confirmed the hyperplastic monocytyosis without immature precursors or pathological lymphoid infiltration, suggesting the diagnosis of CMML type 1 according to WHO classification. CT scan revealed cervical, axillar, mediastinal, abdominal lymph node involvement and moderate hepatosplenomegaly. Node biopsy demonstrated a CD20- clonal lymphocyte population, with the cytologic features of monocytic cells, suggestive for diffuse large B cell lymphoma. Interestingly, a concomitant monocytoid population was also detected. Thereafter, the patient received 6 cycles of CHOP regimen every 28 days. Restaging of patient, performed with CT scan, bone marrow aspiration and biopsy, showed a reduction of spleen size and no evidence of node involvement. Bone marrow examination showed persisting monocytyosis without blast excess and dysplastic features. As expected, therapy against lymphoma did not show any efficacy on CMML but there was no progression of desease. The patient was not furtherly treated for both the old age and poor compliance. One year later, on September 2003, patient developed a disease progression of both lymphoproliferative disorder and CMML as showed by physical examination, bone marrow biopsy, TC scan and laboratory findings. Then he was given combined treatment with Fludarabine (25 mg/m^2 i. v., day 1-3) and Mithoxantrone (8mg/m^2 i. v., day 1) every 28 days for three cycles that were
completed on December 2003. After therapy he achieved a complete remission of the lymphoproliferative disorder and a partial remission of CMML. Patient is presently alive and without treatment, in continuous complete remission for lymphoma but with all the features of chronic myelomonocytic leukemia, without transfusion requirement. Association between lymphoma and CMML is rare. Only three are the published cases until to-day. Two of them are T-lineage lymphomas, and the remaining a breast B-lymphoma. Coexisting untreated lymphoproliferative disease and myelodysplasia has been reported as causal in a series of 1198 patients affected by myelodysplasias (Forlensa, Leukemia and Lymphoma 1996). Only in 5 diagnosed CMMLs concomitant lymphoid and myeloid disease was found. However, all lymphomas were B cell low grade NHLs. This is, at our knowledge, the first report of a B cell high grade NHL coexisting at diagnosis with CMML.

**PO-210**

**ABERRANT SOMATIC HYPERMUTATION OF PROTO-ONCOGENES IN POST-TRANSPLANT LYMPHOPROLIFERATIVE DISORDERS**

Cerri M,1 Capello D,1 Muti G,2 Rambaldi A,2 Paulli M,4 Gloghini A,6 Berra E,1 Deambrogi C, Rossì D,1 Vendramin C,1 Morra E,2 Pasqualucci L,6 Carbone A,5 Gaidano G1

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Post–transplant lymphoproliferative disorders (PTLD) are a heterogeneous group of lymphoproliferations arising in solid organ transplant recipients recieving immunosuppressive therapy. To date, only few molecular lesions of the cellular genome have been associated to the pathogenesis of PTLD. It has been recently shown in diffuse large B-cell lymphoma (DLBCL) of the immunocompetent host and in HIV-non–Hodgkin lymphoma that aberrant somatic hypermutation (SHM) activity can affect multiple genetic loci, including the proto-oncogenes PAX–5, Rho/ITF, PIM–1 and c–MYC. Mutations involve 5’ untranslated regions as well as coding sequences, are independent of chromosomal translocations to the immunoglobulin (Ig) genes and display features and distribution typical of IgV SHM, suggesting that this process is malfunctioning in lymphoma. The knowledge that PTLD derive from GC-related B-cells that have been exposed to the SHM process prompted our analysis of aberrant somatic hypermutation in PTLD. Twenty-five monoclonal B-cell PTLD classified into polymorphic PTLD (P–PTLD; n=5) and monomorphic lymphoma including DLBCL (n=18), and BL/BLL (n=2) formed the basis of our study. Mutional analysis was performed by amplification and direct sequencing of a region spanning up to 1.5 Kb from the transcription start site and previously shown to harbor over 90% of the mutations. Mutations targeting at least one of the 4 proto-oncogenes were found in 7/25 PTLD (28%). All mutated cases were represented by DLBCL (7/18; 38.8%). One single case harbored mutations in more than one gene. PAX–5 was mutated in 4/25 (16%) PPTLD, c–MYC was mutated in 3/25 (12%) PTLD, Rho/ITF was mutated in 1/25 (4%) PTLD, while PIM–1 was not mutated in any case. Mutations were independent of EBV infection since aberrant hypermutation occurred in 3/13 EBV positive PTLD and 4/12 EBV negative PTLD. The mutation frequency of each gene in mutated cases ranged from 0.4 to 8.5x10^-3 bp. Mutations were of somatic origin, as confirmed by analysis of normal DNA from the same patient in selected cases. Mutations were heterozygous and shared features of IgV SHM process. These included: i) the predominance of single base pair substitutions (n=29), with only one deletion; and ii) a preference for transitions (n=16) over transversions (n=13), with a higher than expected transition/transversion ratio (observed=1.23; expected=0.5). In the case of c–MYC, one mutation was located in the coding exons leading to a Ile129Val aminoacid substitution affecting the transactivation domain of the c–MYC protein and carrying potential functional consequences. Based on nonparametric statistical analysis (Kruskal Wallis and Mann–Whitney test with Bonferroni adjustment for multiple comparison), the frequency of aberrant hypermutation in PTLD/DLBCL did not differ from that of AIDS–DLBCL but was lower than that reported in DLBCL of immunocompetent hosts (p<0.05). The frequency of PTLD mutations targeting RGYW motifs (18%) was not of statistical significance. Overall, analogous to DLBCL of the immunocompetent host and HIV-non Hodgkin lymphoma, aberrant SHM may be also implicated in PTLD pathogenesis. Aberrant SHM displays typical molecular features of IgV SHM, suggesting a derivation from abnormal activity of the physiological IgV SHM process. Aberrant SHM may have relevant functional consequences in PTLD development, since it may deregulate gene transcription by targeting regulatory regions or it may alter the biochemical properties of the protein by introducing aminoacid substitutions.

**PO-211**

Not published
PO-212

ANALYSIS OF IGV GENES SUGGESTS THAT MOST POST-TRANSPLANT LYMPHOPROLIFERATIVE DISORDERS DERIVE FROM B-CELLS THAT HAVE FAILED THE GERMINAL CENTRE REACTION

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Posttransplant lymphoproliferative disorders (PTLD) represent a frequent source of morbidity and mortality among solid organ transplant recipients receiving immunosuppression therapy. PTLD are generally of B-cell origin and comprise a histologic spectrum ranging from polyclonal hyperplasia to overt lymphoma. Based on WHO classification, PTLD are classified in early reactive lesions, polymorphic PTLD (P-PTLD) and monomorphic PTLD, comprising diffuse large B cell lymphoma (DLBCL) and Burkitt/Burkitt-like lymphoma (BL/BLL). Although recent studies have elucidated the lymphoma (DLBCL) and Burkitt/Burkitt-like lymphoma (BL/BLL) for usage, mutation frequency and mutation pattern of clonal IgVH and IgVL rearrangements. Two cases showed hybrid Ig VDJ rearrangements: two cases with a V-V fusion rearrangement and one case with a J-J fusion rearrangement, suggesting a failed attempt of heavy chain receptor revision in germin centre (GC) reaction. Despite extensive investigation by multiple PCR strategies, a functional IgVL rearrangement was found in only 25/54 (46.3%) cases. Eleven out of 25 (44.0%) cases harbored IgV kappa rearrangement and 12/25 (48.0%) cases harbored functional IgV lambda rearrangements. Two cases showed the presence of both IgV kappa and IgV lambda functional rearrangements. Among PTLD carrying solely nonfunctional IgVL rearrangements, 7/54 (13.0%) cases showed a crippled rearrangement and 11/54 (20.4%) cases harbored only an out of frame and/or inactivated IgV kappa gene. Inactivation occurred by rearrangement involving the κ-deleting element (KDE). In 11/54 (20.4%) cases, no IgVL rearrangement was identified. Overall, only 23/54 (42.6%) PTLD displayed both a functional IgVH and a functional IgVL rearrangement. Analysis of V, D and J family usage in productive IgVH rearrangements did not show any significant difference with respect to the normal mature B-cell repertoire. Analysis of somatic hypermutation of IgV genes showed the presence of somatically hypermutated IgVH and/or IgVL genes in 45/54 PTLD (83.3%). Conversely, IgV rearrangements of 9/54 (16.6%) PTLD were in germline configuration, suggesting a derivation from B-cells that have not experienced the GC-reaction. Among mutated cases, the average mutation frequency was 8.83% (median 8.43%, range 2.10%-24.1%) for IgVH genes and 7.37% (median 6.71%, range 2.30%-26.0%) in IgVL genes. Thirty-two cases (74.4%) showed highly mutated (mutation frequency >6%) IgVH and/or IgVL genes, a condition that, in normal B-cell, results in lower affinity for antigen and apoptosis. Analysis of the distribution of replacement and silent mutations in functional IgVH and/or IgVL sequences showed tendency to conserve FR sequences and maintain antigen binding in 20/34 (58.8%) cases. A higher than expected number of CDR replacement mutations, suggesting selection for high affinity antigen binding, occurred in 14/34 (41.2%) cases. Overall, our data suggest that most PTLD arise from B-cells that have experienced the GC-reaction and frequently display impaired B-cell receptors (BCR). Since a functional receptor is required for the survival of normal B-cells during their transit throughout the GC, PTLD development seems frequently associated with rescue from apoptosis and expansion of B-cells that have failed the GC-reaction. One mechanism might involve EBV infection, with the expression of the oncoproteins LMP1 and/or LMP-2A that mimic the signals normally generated by CD40 and BCR. Notably, virtually all PTLD with nonfunctional IgVH and/or IgVL rearrangement analyzed in this study, carried EBV infection.

PO-213

HYPERMETHYLATION OF THE 5’ CPG ISLAND OF THE FHIT (FRAGILE HISTIDINE TRIAD) GENE THROUGHOUT THE SPECTRUM OF B-CELL NEOPLASIA MALIGNANCIES AND AIDS-NHL

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Haematologica vol. 89[suppl. n. 6]:september 2004
DNA methylation of CpG sites in the promoter regions of genes is a frequent acquired epigenetic event in the pathogenesis of many human cancers. This modification has important regulatory effects causing loss of gene expression. To date, studies of aberrant promoter methylation in B-cell lymphoid neoplasia have revealed the occurrence of epigenetic alterations of several genes with multiple functions. FHIT (Fragile Histidine Triad) is considered a putative tumor suppressor gene located at the aphidicolin FRA3B site of chromosome 3p14.2. The FHIT gene encodes a triphosphate hydrolase that catalyzes the hydrolysis of intracellular dinucleoside polyphosphates and is involved in control of cell growth and apoptosis by modulating the intracellular concentration of polyphosphates. Here we analyzed 404 B-cell malignancies of the immunocompetent host and 97 tumor samples of AIDS-related non-Hodgkin lymphoma (AIDS-NHL) by methylation-specific polymerase chain reaction (MSP) of the FHIT gene. Among precursor B-cell neoplasms, FHIT hypermethylation occurred in 6/21 (28.6%) acute lymphoblastic leukemias (ALL). Among indolent lymphoproliferative disorders, FHIT hypermethylation was restricted to 14/97 (14.4%) B-cell chronic lymphocytic leukemia (B-CLL), 1/11 (9.1%) marginal zone lymphoma (MZL), 3/10 (30.0%) MALT lymphoma, 2/18 (11.1%) follicular lymphoma (FL), 1/6 (16.7%) hairy cell leukemia (HCL), and 2/16 (12.5%) plasmacytoid lymphoma (LPL). Among aggressive B-cell lymphomas, FHIT hypermethylation occurred in 18/135 (13.3%) diffuse large B-cell lymphomas (DLBCL), and in 8/40 (20.0%) Burkitt’s lymphomas; in particular, among DLBCL, only few sub-groups were interested by this aberrant mechanism, including 12/73 (16.4%) systemic nodal B-DLCL, 2/5 (40%) CD30+ anaplastic B-DLCL, 1/7 (14.3%) CD5+ B-DLCL, and 3/16 (18.8%) primary central nervous system lymphomas (PCNSL). Primary mediastinal DLBCL (n=9), primary splenic DLBCL (n=6), and DLBCL transformed from FL (n=9) or MALT-NHL (n=9) showed no FHIT hypermethylation. Also, FHIT hypermethylation was absent in primary effusion lymphoma (PEL) (0/4), and in mantle cell lymphoma (0/18). Finally, FHIT hypermethylation occurred in a fraction of multiple myeloma (MM) (6/28; 21.4%). With respect to AIDS-related non-Hodgkin’s lymphomas (AIDS-NHL), that rate of FHIT hypermethylation rate was similar in all the categories analyzed. In particular, FHIT hypermethylation occurred in 7/48 (14.6%) AIDS-DLBCL, 5/27 (18.5%) AIDS-BL, and 1/15 (6.7%) AIDS-PEL. No FHIT hypermethylation was detected in AIDS-BLL (Burkitt’s like lymphoma; 0/7). Overall, these data indicate that inactivation of FHIT gene by aberrant hypermethylation may have a role in the pathogenesis of a fraction of B-cell neoplasms of immunocompetent and HIV-infected patients.
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VIII Congress of the Italian Society of Experimental Hematology, Pavia, September 14-16, 2004