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3rd Congress of the Italian Society of Experimental Hematology

Udine-Grado, September 22-24, 1994

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Udine-Grado, September 22-24, 1994

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ASSESSMENT OF MOLECULAR STATUS IN LONG SURVIVAL ACUTE PROMYELOCYTIC LEUKEMIA PATIENTS BY REVERSE TRANSCRIPTION POLYMERASE CHAIN REACTION OF PML-RAR FUSION MRNA. C. Remiddi, G. Martinelli, G. Visani, P. Farabegoli, N. Testoni, S. Manfroi, D. Russo, M. Amabile, A. Zaccaria, L. Salini, M. Salvucci, A. Di Nota, M. Barassi, A. Cenacchi, S. Tura

ATRA+ANTRACYCLINES IN PATIENTS WITH ACUTE PROMYELOCYTIC LEUKEMIA: HEMOSTATIC ASPECTS. L. Gugliotta, F. Nocentini, L. Catani, N. Vianelli, A. Cenacchi, S. Baravelli, G. Visani, S. Tura

IN PROMYELOCYTIC BLAST CRISIS OF CHRONIC GRANULOCYTIC LEUKEMIA DUAL COLOR FISH DETECTS BOTH THE T(9;22) AND THE T(15;17). P. Bernasconi, M. Boni, P. M. Cavigliano, D. Troletti, F. Passamonti, C. Castagnola, E. Morra, E. P. Alessandrino, G. Biaggi, C. Bernasconi

122-133 SIMULTANEOUS SESSION 13 – Hemopoietic progenitors

INTERACTION OF STEM CELL FACTOR (SCF), INTERLEUKIN 3 (IL3) AND BASIC FIBROBLAST GROWTH FACTOR (bFGF) IN THE REGULATION OF NORMAL AND PRELEUKEMIC MEGAKARYOCYTOPOIESIS. W. Piacibello, F. Sanavio, L. Garetto, A. Severino, L. Fubini, M. Aglietta

ALL-TRANS RETINOIC ACID (ATRA) POTENTIATES MEGAKARIOCYTE COLONY FORMATION: IN VITRO AND IN VIVO EFFECTS AFTER ADMINISTRATION TO ACUTE NON LYMPHOID LEUKEMIA PATIENTS. G. Visani, G. Zauli, P. Tosi, E. Ottaviani, D. Gibellini, C. Pagliarini, S. Manfroi, S. Tura

PROLIFERATIVE POTENTIAL AND PHENOTYPE OF HEMOPOIETIC PROGENITORS COLLECTED BY LEUKAPHERESES FROM SEVERE APLASTIC ANEMIA (SAA) PATIENTS AFTER PROLONGED G-CSF TREATMENT. G. Piaggio, M. Podestà, O. Figari, F. Benvenuto, J. Tong, A. Bacigalupo

NORMAL AND LEUKEMIC CD34-POSITIVE PROGENITORS FROM CHRONIC MYELOGENOUS LEUKEMIA PATIENTS HAVE A DIFFERENTIAL CAPACITY TO ADHERE TO ALLOGENEIC STROMA. G. P. Dotti, C. Carlo-Stella, L. Mangoni, G. Piovani, D. Garau, C. Almici, V. Rizzoli

ANTIPROLIFERATIVE ACTION OF THE PROTEIN-TYROSINE KINASE INHIBITOR GENISTEIN ON NORMAL AND LEUKEMIC HEMATOPOIETIC PROGENITOR CELLS. E. Regazzi, C. Carlo-Stella, L. Mangoni, M. T. Rizzo, D. Garau, V. Rizzoli

HIGH-DOSE CHEMOTHERAPY FOLLOWED BY ERYTHROPOIETIN PLUS G-CSF FOR STEM CELL MOBILIZATION. P. Leoni, A. Olivieri, M. Offidani, I. Cantori, L. Ciniero, P. Scalari, C. Masia, M. Montroni

SEQUENTIAL ADMINISTRATION OF INTERLEUKIN-3 (IL-3) AND GRANULOCYTE-MACROPHAGE COLONY-STIMULATING FACTOR (GM-CSF) AFTER HIGH-DOSE CYCLOPHOSPHAMIDE (HDCY). A. Ballestrero, F. Ferrando, P. Basta, A. Garuti, P. Stura, M. Gobbi, R. Ghio, F. Brema, F. Patrone

CORRELATION BETWEEN THROMBOMODULIN (TM) PLASMA LEVEL AND PERIPHERAL BLOOD CD34+ CELL NUMBER AFTER HIGH DOSE (HD) CHEMOTHERAPY. A. Manna, S. Testa, R. Carpanelli, F. Kang, S. Macchi, A. Porcellini

IN VITRO EXPANSION OF CD34+ HEMOPOIETIC STEM CELLS. F. Buscemi, A. Santoro, S. Vasta, M. Pampinella, T. Fiandaca, P. Catania, A. Indovina, R. Scimè, I. Majolino

EXPANSION OF EARLY HEMATOPOIETIC PROGENITORS FROM LEUKAPHERESES WITH A COMBINATION OF FOUR CYTOKINES WITHOUT CD 34+ CELLS PURIFICATION. A. Olivieri, S. Rupoli, C. Masia, I. Cantori, L. Ombrosi, A. R. Scortechini, S. Mancini, P. Leoni

ANALYSIS OF HEMATOPOIETIC PROGENITOR CELLS FROM UMBILICAL CORD BLOOD AFTER 3 WEEK SUSPENSION CULTURE WITH DIFFERENT GROWTH FACTORS. A. Balduini, M. Bonfichi, C. Brera, M. Savio, P. Bernasconi, E. P. Alessandrino, G. Pagnucco, F. Polatti, C. Bernasconi

PROLIFERATIVE ACTIVITY OF HEMATOPOIETIC PROGENITOR CELL FROM UMBILICAL CORD BLOOD (UCB) WITH IL3 AND GM-CSF. M. Bonfichi, C. Brera, M. Savio, A. Balduini, E.P. Alessandrino, P. Bernasconi, G. Pagnucco, F. Polatti, C. Bernasconi

134-145 SIMULTANEOUS SESSION 14 – Growth factors and receptors

IDENTIFICATION AND CHARACTERIZATION OF ERYTHROPOIETIN RECEPTORS ON THE HUMAN HEP3B HEPATOMA CELL LINE. A.M. Vannucchi, A. Ieri, A. Grossi, S. Linari, D. Rafanelli, P. Rossi Ferrini

FLOW CYTOMETRY DETECTION OF GM-CSF-R IN ACUTE MYELOID LEUKEMIA AND MYELODISPLASTIC SYNDROMES. F. Lanza, G.M. Rigolin, S. Moretti, A. Latorraca, B. Castagnari, R. Balsano, G.L. Castoldi

EFFECTS OF THE c-kit LIGAND AND OTHER GROWTH FACTORS ON BLAST CELL PROLIFERATION OF ACUTE MYELOID LEUKEMIA PATIENTS: SYNERGISTIC INTERACTION BETWEEN SCF AND PIXY 321 AND ENHANCEMENT OF ARA-C CYTOTOXICITY. A. Tafuri, L. De Felice, M.G. Mascolo, T. Valentini, M.T. Petrucci, F. Mandelli

EFFECTS OF SCF, IL-3 AND ERYTHROPOIETIN ON THE DIFFERENTIATION OF A HUMAN ERYTHROLEUKEMIA CELL LINE (TF-1). A. Grossi, P. Bacci, A.M. Vannucchi, R. Caporale, D. Rafanelli, S. Eridani,P. Rossi Ferrini

TGF-β3 INHIBITS HUMAN PRIMITIVE HEMATOPOIETIC PROGENITORS. IN-VITRO EFFECTS OF THE EARLY ACTING CYTOKINES IL-11, SCF AND IL-9. M. Fogli, A. Fortuna, S. Tura, R.M. Lemoli

TNF RECEPTORS ARE EXPRESSED BY ACUTE LEUKEMIA CELLS AND ARE ASSOCIATED WITH INCREASED SERUM LEVELS OF THE CORRESPONDING SOLUBLE MOLECULES. C. Tecchio, A. Rigo, L. Morosato, F. Vinante, R. Zanotti, G. Nadali, M.M. Ricetti, M. Chilosi, H. Gallati, G. Pizzolo

EXPRESSION AND FUNCTIONAL ROLE OF C-KIT LIGAND (SCF) IN HUMAN MULTIPLE MYELOMA (MM) CELLS. A. Fortuna, A. Grande, L. Bonsi, M. Fogli, M. Amabile, M. Cavo, S. Ferrari, S. Tura, R. M. Lemoli

ARACHIDONIC ACID MEDIATES INTERLEUKIN-1 AND TUMOR NECROSIS FACTOR-INDUCED C-JUN EXPRESSION IN STROMAL CELLS BY A PROTEIN KINASE C-INDEPENDENT PATHWAY. M. T. Rizzo, H. S. Boswell, D. English, L. Mangoni, C. Carlo-Stella, V. Rizzoli

MEMBRANE-BOUND, SOLUBLE IL-2 RECEPTORS (IL-2R) AND LEVELS OF IL-1α, IL-2 AND IL-6 IN PATIENTS WITH HEMATOLOGICAL MALIGNANCIES. G. Mantovani, A. Macciò, P. Lai, S. Esu, L. Curreli, A. Bianchi, B. Lampis, E. Turnu, A. Balestrieri, G. S. Del Giacco

EFFECTS OF IL-4 AND IL-7 ON THE GROWTH AND DIFFERENTIATION OF NORMAL B CELL PRECURSORS. R. Consolini, A. Legitimo

INTERLEUKIN 8 MAY EXERT A FUNCTIONAL AUTOCRINE/PARACRINE ROLE IN B-CELL CHRONIC LYMPHOCYTIC LEUKEMIA BY PROLONGING THE SURVIVAL OF THE NEOPLASTIC CLONE. P. Francia di Celle, S. Mariani, L. Riera, A. Carbone, R. Foà

IN VITRO EFFECTS OF rhIL-2 ON MDS BONE MARROW: ANALYSIS OF CLONOGENIC GROWTH AND CYTOKINES PRODUCTION. C. Clerici, B. Sarina, C. Cattaneo, I. Silvestris, M. Pomati, A. Cortelezzi, A. T. Maiolo

146-157 SIMULTANEOUS SESSION 15 – Lymphoproliferative disease and lymphoma

IN VIVO ANTITUMOR ACTIVITY OF ANTI-CD22/RIPs 1 IMMUNOTOXINS AGAINST DISSEMINATED DAUDI B-CELL LYMPHOMA IN SCID MICE. A. Terenzi, L. Pasqualucci, L. Flenghi, A. Bolognesi, M. F. Martelli, F. Stirpe, B. Falini

OVER-UTILIZATION OF IMMUNOGLOBULIN VH4 GENE FAMILY IN AIDS-RELATED NON-HODGKIN LYMPHOMA (AIDS-NHL). V. Cilli, D. Petroni, E. Gottardi, C. Pastore, U. Mazza, A. Carbone, G. Gaidano, G. Saglio

ANALYSIS OF BCL 2 EXPRESSION IN NEOPLASTIC AND REACTIVE SKIN LESIONS: A HINT TO THE PATHOGENESIS OF CUTANEOUS T CELL LYMPHOMA? F. Vianello, T. Tison, C. Giacon, P. Radossi, A. Poletti, A. Girolami, F. Dazzi

APOPTOSIS INDUCTION WITH THREE NUCLEOSIDE ANALOGS OF FRESHLY ISOLATED B-CHRONIC LYMPHOCYTIC LEUKEMIA CELLS. M. Buzzi, P.L. Zinzani, P. Tosi, G. Visani, E. Ottaviani, P. Farabegoli, G. Martinelli, S. Tura

IN VITRO STUDY OF CHLORAMBUCIL- AND PURINE ANALOGS-INDUCED CYTOTOXICITY IN CHRONIC LYMPHOCYTIC LEUKEMIA. CORRELATION WITH HEMATOLOGICAL FEATURES. G. Messina, F. Morabito, I. Callea, A. Pontari, C. Stelitano, M. Brugiatelli, F. Nobile

CLINICAL SIGNIFICANCE OF CIRCULATING SOLUBLE ANTIGENS (sCD4, sCD8, sCD23, sCD25, sCD30, sCD54), A NEW CLASS OF TUMOR MARKERS, IN CHRONIC LYMPHOPROLIFERATIVE DISORDERS (CLD): THE SIX YEARS EXPERIENCE OF A SINGLE CENTRE. P. Musto, R. Matera, M.M. Minervini, P. D'Arcangelo, A. La Sala, N. Di Renzo, M. Dell'Olio, C. Bodenizza, A. Falcone, P. Scalzulli, G. D'Arena, M. Carotenuto

DISAPPEARANCE OF PCR-DETECTABLE LYMPHOMA CELLS IN PERIPHERAL BLOOD AND BONE MARROW CELL HARVESTS AFTER HIGH-DOSE SEQUENTIAL CHEMOTHERAPY. P. Corradini, M. Astolfi, C. Voena, P. Bondesan, C. Cherasco, M. Boccadoro, C. Tarella, A. Pileri

PILOT STUDY USING HDS REGIMEN WITH INTENSIFIED DEBULKYING PRETREATMENT IN LOW/INTERMEDIATE GRADE NON-HODGKIN'S LYMPHOMA AT DIAGNOSIS. C. Tarella, D. Caracciolo, P. Gavarotti, P. Corradini, F. Zallio, C. Castellino, A. Pileri

METHOTREXATE+AZT IN HIGH-GRADE HIV-RELATED NON-HODGKIN LYMPHOMAS . F. Gherlinzoni, P. Tosi, G. Visani, P. Mazza, P.L. Zinzani, M.C. Miggiano, O. Coronado, E. Ricchi, P. Costigliola, E. Raise, F. Chiodo, S. Tura

RANDOMIZED MULTICENTER TRIAL WITH OR WITHOUT GRANULOCYTE COLONY-STIMULATING FACTOR AS ADJUNCT TO INDUCTION VNCOP-B TREATMENT OF ELDERLY HIGH-GRADE NON-HODGKIN'S LYMPHOMA. P. L. Zinzani, S. Storti, E. Aitini, P. Fattori, L. Moretti, P. Gentilini, L. Guardigni, V. M. Lauta, V. Pavone, A. De Renzo, A. Cuneo, G. Storti, G. Leone, F. Dammacco, V. Liso, B. Rotoli, E. Volpe, F. Gherlinzoni, M. Bendandi, S. Tura

FIRST RESULTS FROM A POPULATION CASE-CONTROL STUDY ON CHRONIC LYMPHOCYTIC LEUKEMIAS AND NON HODGKIN'S LYMPHOMAS ACCORDING TO HISTOLOGIC TYPE IN FARMING-ANIMAL BREEDING. D. Amadori, C. Milandri, O. Nanni, F. Falcini, A. Callea, P. Vignutelli, P. Gentilini

LYMPHOMAS AND LEUKEMIAS: INCIDENCE DATA IN UU. SS. LL. 35-37-38 FOR YEARS 1986-91. P. Gentilini, C. Milandri, P. Vignutelli, F. Falcini, O. Nanni, F. Martini, P. Serra

158-163 PLENARY SESSION 3 – Chronic myeloid leukemia

EXPRESSION OF HEMATOPOIETIC GROWTH FACTOR AND OTHER CYTOKINE mRNAs DURING THE COURSE OF CHRONIC MYELOID LEUKEMIA. A. Tabilio, F. Falcinelli, M. Onorato, F. Falzetti, C. Giannoni, R. Ciurnelli, S. Covalovo, M. F. Martelli

POSITIVE SELECTION OF CD34+ CELLS: A SHORT REVIEW OF THE METHODS CURRENTLY AVAILABLE FOR EXPERIMENTAL AND CLINICAL USE WITH PARTICULAR FOCUS ON IMMUNOMAGNETIC BEADS AND CHYMOPAPAIN. F. Silvestri, C. Savignano, C. Rinaldi, G. Trani, D. Damiani, F. Biffoni, M. Baccarani

CHARACTERIZATION AND SELECTION OF BENIGN STEM CELLS IN CHRONIC MYELOID LEUKEMIA. R.M. Lemoli

CML: PH-NEGATIVE CELLS COLLECTED AFTER CHEMOTHERAPY CONTAINING LTC-ICS ARE ABLE TO RESTORE AND SUSTAIN POLYCLONAL HEMATOPOIESIS AFTER AUTOGRAFTING. F. Frassoni, M. Podestà, D. Giordano, E. Pungolino, N. Pollicardo, C Parodi, M Sessarego, G. Piaggio, M. R. Ferrero, M. Soracco, G. Valbonesi, R. Hoffman, G Saglio, A. M. Carella

MODULATION OF CELL KINETICS AND IN VITRO CELL GROWTH OF CML CD34+ PROGENITORS INDUCED BY p53 ANTISENSE OLIGONUCLEOTIDES. F. Lanza, G.L. Castoldi

AUTOLOGOUS BONE MARROW TRANSPLANTATION AFTER IN VITRO PURGING WITH BCR-ABL ANTISENSE OLIGODEOXYNUCLEOTIDES FOR PATIENTS WITH CHRONIC MYELOID LEUKEMIA IN ADVANCED PHASE. P. de Fabritiis, E. Montefusco, A. Lisci, S. De Propris, M. Mancini, S. Buffolino, P. Pontis, S. Amadori, B. Calabretta, F. Mandelli

164-188 POSTER SESSION 1 – Hemopoietic progenitors and growth factors

COLLECTION OF PRIMITIVE AND COMMITTED CLONOGENIC CELLS FOLLOWING MOBILIZATION OF CIRCULATING PROGENITORS WITH CHEMOTHERAPY PLUS G-CSF OR G-CSF ALONE. L. Cottafavi, L. Mangoni, G. P. Dotti, G. L. Cavanna, L. Craviotto, C. Caramatti, C. Almici, C. Carlo-Stella, V. Rizzoli

MOBILIZATION OF CIRCULATING HEMATOPOIETIC PROGENITOR CELLS (CHPC) BY STANDARD-DOSE CHEMOTHERAPY + G-CSF IN BREAST CANCER PATIENTS. R. Ghio, E. Balleari, C. Bason, L. Del Mastro, O. Garrone, G. Massa, . G. Melioli, W. Pasquetti, R. Rosso, M. Venturini

PERIPHERAL BLOOD STEM CELL AUTOTRANSPLANT FOLLOWED BY GM-CSF: IMMUNOLOGICAL PROFILE. A. M. Liberati, M. Schippa, D. Adiuto, M. Cecchini, L. Fedeli, I. Sabalic, M. Zuccaccia, F. Di Clemente, S. Mancini, S. Cinieri

HIGHLY EFFICIENT PURIFICATION OF CD34+ HEMATOPOIETIC PROGENITOR CELLS BY HIGH-GRADIENT MAGNETIC CELL SORTING. C. Bason, A. Garuti, E. Balleari, A. Ballestrero, M. Dress, F. Ferrando, R. Ghio, G. Melioli, F. Patrone

EFFECT OF BONE MARROW STROMAL CELLS ON OSTEOCYTIC CELLS LINES. G. Lisignoli, M. C. G. Monaco, A. Degrassi, D. Damiani, M. Michieli, S. Lavaroni, M. Scarbolo, S. Formisano, A. Facchini

IN VITRO GROWTH FRACTION EVALUATION OF LYMPHOID BLAST CELLS FOLLOWING EXPOSITION TO GM-CSF. A. Camera, S. Rocco, M. R. Villa, F. Alfinito, A. Ruggiero, S. Pepe, B. Rotoli

PLASMA SOLUBLE STEM CELL FACTOR LEVELS IN PATIENTS WITH ACUTE NON-LYMPHATIC LEUKEMIA. M. Pomati, F. Bamonti-Catena, B. Sarina, A. T. Maiolo

DETERMINATION OF ERYTHROPOIETIN LEVELS AFTER PERIPHERAL BLOOD PROGENITOR CELL TRANSPLANTATION (PBPCT): COMPARISON WITH AUTOLOGOUS BONE MARROW TRANSPLANTATION. P. Salutari, S. Sica, A. Di Mario, S. Rutella, U. Testa, R. Martucci, C. Peschle, G. Leone

INTERLEUKIN 1 AND GRANULOCYTE MACROPHAGE COLONY STIMULATING FACTOR REDUCE THE IN VITRO INHIBITORY EFFECT OF AN AZIDOTHYMIDINE INTERFERON α COMBINATION ON HUMAN HEMOPOIETIC PROGENITORS. G. Castello, G. Mela, A. Cerruti, M. Mencoboni, R. Lerza

IL-6 SERUM LEVELS IN PATIENTS AFFECTED BY HAEMATOLOGIC MALIGNANCIES: CORRELATION WITH NEUTROPENIA AND INFECTIONS. S. Rupoli, G. Pomponio, M. Fratini, A. Cinciripini, P. Paoletti, A. Recchioni, F. Federiconi, P. Leoni

IN VITRO EFFECTS OF rhSCF ON MEGAKARYOCYTIC COLONIES IN MDS PATIENTS. M. Di Stefano, B. Sarina, C. Cattaneo, I. Silvestris, D. Soligo, A. Cortelezzi, L. Bonsi, A.T. Maiolo

LONG -LASTING HEMATOLOGICAL REMISSION OF REFRACTORY ANEMIA AFTER A SHORT COURSE OF GRANULOCYTE-MACROFAGE COLONY STIMULATING FACTOR (GM-CSF): A CASE REPORT. F. Silvestri, L. Virgolini, R. Fanin, M. Baccarani

THE GROWTH INHIBITION OF HUMAN LEUKEMIC BLASTS BY QUERCETIN INVOLVES THE INDUCTION OF TRANSFORMING GROWTH FACTOR β. L.M. Larocca, L. Teofili, M.S. Iovino, M. Piantelli, N. Maggiano, S. Sica, F. O. Ranelletti , G. Leone

INDUCTION OF FETAL HEMOGLOBIN BY BUTYRATE ANALOGUES IN ERYTHROID LIQUID CULTURE FROM HUMAN PERIPHERAL BLOOD STEM CELLS. M.D. Cappellini, I. Stefanoni, C. Tomaselli, P. Bianchi, A. Ronchi, S. Ottolenghi, G. Fiorelli

RECOMBINANT ERYTROPOIETIN FOR THE TREATMENT OF ANEMIA IN SELECTED PATIENTS WITH MYELODISPLASTIC SYNDROMES. C. Caramatti, L. Mangoni, C. Carlo-Stella, C. Almici, L. Craviotto, G. L. Cavanna, V. Rizzoli

SERUM ERYTHROPOIETIN INCREASE IN ABSENCE OF ANEMIA FOLLOWS ADJUVANT THERAPY WITH 5FU-LV. A. Cerruti, G. Castello, E. Balleari, R. Lerza, G. Bogliolo, I. Pannacciulli

REGULATORY ACTION OF PROLACTIN ON THE IN VITRO GROWTH OF CD34+ve HUMAN HEMOPOIETIC PROGENITOR CELLS. G. Bellone, P. Astarita, C. Cravioglio, S. Silvestri, L. Matera, G. Emanuelli

EFFECT OF PROCAINE HYDROCHLORIDE ON MYELOTOXICITY INDUCED BY CARBOPLATIN IN NORMAL MICE. R. Lerza, M. Esposito, M. Mencoboni, G. Castello, M. Vannozzi, M. Viale, A. Cerruti, G. Bogliolo

MEGAKARYOCYTIC PROGENITORS IN PERIPHERAL BLOOD AFTER SALVAGE CHEMOTHERAPY INCLUDING CARBOPLATINUM AND G-CSF ADMINISTRATION IN PATIENTS WITH RESISTANT LYMPHOMA. A. Di Mario, L. Teofili, E. Ortu La Barbera, S. Rutella, S. Sica, P. Salutari, G. Leone

ATYPICAL EXPRESSION OF LEUKOCYTE COMMON ANTIGEN (CD45) ISOFORMS ON PERIPHERAL BLOOD PROGENITOR CELLS. S. Rutella, E. Ortu-La Barbera, C. Rumi, S. Sica, G. Leone

COUNTERFLOW CENTRIFUGAL ELUTRIATION OF G-CSF MOBILIZED CELLS: IN VITRO STUDY OF CD34+ CELLS. L. Teofili, M. S. Iovino, E. Ortu La Barbera, A. Di Mario, L. Pierelli, G. Menichella, C. Rumi, G. Leone

EFFECT OF A NEW CHEMOTHERAPEUTIC REGIMEN (MiCMA) ON THE MOBILIZATION OF CIRCULATING HEMATOPOIETIC PROGENITORS. E. Ortu La Barbera, S. Rutella, L. Teofili, C. Rumi, A. Di Mario, G. Leone

THE IN VITRO ACTIVITY OF SOME URINARY POLYPEPTIDES ON THE PERIPHERAL CFU OF NORMAL AND LEUKEMIC SUBJECTS IN COMPARISON WITH G- AND GM-CSF AND IL3. J. Mazzucchelli, M.L. Rolandi, G. Fossati, A. Notario

COMPARISON OF GM-CSF-INDUCED IMMUNOLOGICAL EFFECTS AFTER CYCLOPHOSPHAMIDE OR CARBOPLATINUM. A.M. Liberati, M. Schippa, D. Adiuto, M. Cecchini, L. Fedeli, R. Palumbo, A. R. Betti, F. Di Clemente, S. Mancini, S. Cinieri.

PRODUCTION OF GRANULOCYTE-COLONY STIMULATING FACTOR (G-CSF) BY NEOPLASTIC B LYMPHOCYTES FROM B-CLL PATIENTS. A. Corcione, V. Pistoia

189-196 POSTER SESSION 2 – Bone marrow transplantation

PRE-HARVEST ABSOLUTE MONONUCLEAR CELL (MNC) COUNT ON BONE MARROW (BM) ASPIRATE CORRELATES WITH MNC YIELD IN AUTOLOGOUS BM HARVESTS. C. Rinaldi, C. Savignano, F. Silvestri, A. Geromin, M. Cerno, R. Fanin, M. Baccarani, F. Biffoni

BONE MARROW HARVESTS: EFFECT OF FILTRATION ON HAEMOPOIETIC CELL RECOVERY. C. Savignano, C. Feruglio, C. Rinaldi, S. Lavaroni, F. Silvestri, F. Venturelli, A. Degrassi, P. G. Sala, F. Biffoni, S. Formisano, M. Baccarani

PERFORIN AND GRANZYME B AS PREDICTIVE MARKERS FOR GVHD AFTER BONE MARROW TRANSPLANTATION: PRELIMINARY DATA. M. Savio, M. Bonfichi, R. Nano, M. Baiocchi, C. Brera, E. P. Alessandrino, P. Bernasconi, E. Capelli, C. Bernasconi

A RAPIDE PROGRESSIVE PULMONARY FAILURE IN A YOUNG PROMYELOCYTIC LEUKEMIA PATIENT AFTER ALLOGENEIC BONE MARROW TRASPLANTATION. A. Geromin, R. Fanin, G. Barillari, M. Cerno, M. Baccarani

“FINGERPRINTING” OF HLA-CLASS I AND II GENES FOR IMPROVED SELECTION OF RELATED AND UNRELATED BONE MARROW DONOR. G. Martinelli, P. Farabegoli, M. Buzzi, G. Panzica, A. Zaccaria, V. Mantovani, G. Bandini, E. Calori, N. Testoni, G. Rosti, M. Bragliani, R. Conte, S. Tura

EFFECT OF BONE MARROW TRANSPLANTATION ON THE DAYTIME MELATONIN CIRCULATING LEVELS. S. Guidi, F. Perfetto, G. Guidi, A. Piluso, A. Bosi, I. Farhad, R. Tarquini

G-CSF AND IL-3 COMBINATION ACCELERATES HEMATOPOIETIC RECOVERY AFTER ABMT FOR LYMPHOMA PATIENTS. R.M. Lemoli, G. Rosti, M.C. Miggiano, F. Gherlinzoni, A. Fortuna, M. R. Motta, S. Rizzi, G. Visani, S. Tura

SALVAGE CHEMOTHERAPY AND G-CSF ADMINISTRATION FOLLOWED BY AUTOLOGOUS PERIPHERAL BLOOD PROGENITOR CELLS TRANSPLANTATION IN PATIENTS WITH RESISTANT LYMPHOMA. S. Sica, P. Salutari, A. Di Mario, B. Etuk, P. Chiusolo, R. Marra, L. Teofili, L. Pierelli, G. Leone

197-207 POSTER SESSION 3 – Drug resistance

IMMUNOCYTOCHEMICAL AND IMMUNOELECTRON-MICROSCOPICAL IDENTIFICATION OF P-170 IN RAT PERITONEAL MAST CELLS. L. Travan, E. Crivellato, L. Candussio, F. Bartoli Klugmann, G. Decorti, C. Melli, A. Michelutti

EVALUTATION OF MULTI-DRUG RESISTANCE IN PEDIATRIC LEUKEMIAS. A. Sommaggio, M. G. Cocito, M. P. Albergoni, G. Basso

CLINICAL RELAVANCE OF GLUTATHIONE-S-TRANSFERASE AND MULTIDRUG RESISTANCE IN B-CLL. R. Testi, D. Di Simone, F. Caracciolo, E. Capochiani, M. Petrini

INTERLEUKIN-2 AND ALPHA-INTERFERON MODULATION OF CHLORAMBUCIL- AND PURINE ANALOGS-INDUCED CYTOTOXICITY IN CHRONIC LYMPHOCYTIC LEUKEMIA. F. Morabito, I. Callea, G. Messina, G. Irrera, I. Vincelli, V. Callea, P. Iacopino, F. Nobile

MDR-RELATED P170 GLYCOPROTEIN MODULATES THE ANTILEUKEMIC ACTIVITY OF HOMOHARRINGTONINE. D. Russo, L. Infanti, A. Michelutti, C. Melli, A. Candoni, M. Cerno, F. Salmaso, F. Zaja, M. Baccarani

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001

MECHANISMS OF ACTION OF INTERFERONS

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The biological response to IFNs is initiated by cell surface receptor binding and relies on the co-ordinated activation and repression of a set of specific genes. IFNs α/β (type I) and γ (type II) bind to distinct cell surface receptors, stimulating distinct (although partially overlapping) intracellular pathways which finally result in tyrosine phosphorylation of cytoplasmic transcription factors. IFN-α stimulates phosphorylation and nuclear localization of the 84/91Kd and 113Kd subunits of latent ISGF3 (interferon stimulated gene factor 3), which combine with the 48Kd DNA-binding subunit to bind regulatory elements of IFN-α-responsive genes. IFN-γ activates p91 alone, inducing IFN-γ-responsive genes through a distinct DNA element. Evidences have been provided that the tyrosine kinase Tyk2 is involved in IFN-α signalling as the related Jak2 protein in IFN-γ signalling. Other signalling proteins have also been implicated. This degree of knowledge, however, has been of little help in understanding the molecular basis of IFN therapeutic activity in many hematologic diseases, as the cyto-reductive effect exerted by IFN-α on the Ph-positive clone of most CML patients. In vitro studies have indicated that IFN-α might function by: i) selective toxicity against the leukemic clone; ii) intensification of immuno-surveillance; iii) modulation of the bone-marrow microenvironment. Recent data obtained in our lab suggest that the *in vivo* antiproliferative effect exerted by IFN-α on the Ph-positive clone is associated with a selective reduction of the amount of Bcr/Abl transcripts present in the leukemic cells. However, at present it is not clear whether this could be a direct effect induced by IFN-α on the Bcr/Abl rate of transcription or it is mediated through the action of other cytokines.

002

SELECTION AND PURIFICATION OF HEMATOPOIETIC STEM CELLS

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A stem cell is a cell able to reconstitute lympho- myelopoiesis in a lethally irradiated host. Stem cell properties have been defined at the antigenic (CD34, CD38, CD45RA, HLA-DR, Thy1), physical (light scatter, stroma adherence) and functional level (mafosfamide resistance, Rhodamine-123 uptake). In vitro and in vivo assays allowing determination of the stem-ness potential of hematopietic cells have been established. Growth factors and culture systems preferentially affecting stem cell self-renewal or commitment are under investigation. Experimental protocols combining positive and negative selection are available for the isolation and manipulation of stem cell subpopulations. Positive and negative selection is increasingly used to achieve the goal of decontaminating autologous hematopoietic cells.

Negative selection. Three major approaches usually resulting in a 3-6 log cell kill in vitro have been used for in vitro removal of leukemic cells. These approaches include: (1) pharmacological treatment, (2) monoclonal antibodies, (3) in vitro culture. Although retrospective analysis suggest that purging by negative selection might have a beneficial effect on clinical outcome, the results of prospective trials are still required.

Positive selection. Technologies (fluorescence-activated cell sorting, avidin-biotin columns, antibody-coated flasks, immunomagnetic beads) for large scale purification of CD34+ cells are available. Positive selection can be used as purging method in CD34- malignancies, such as multiple myeloma where positive selection results in 2-5 log depletion of CD19+ cells. In CD34+ malignancies, additional selection steps for or against other markers are required. In chronic myelogenous leukemia (CML) stroma adherence or HLA-DR expression have been proposed. Whether or not the phenomenon of post-transplant clonal dominance might be affected by the use of highly enriched CD34-positive cells and might eventually influence the long-term outcome of autografting is still unclear.

Experimental approaches. (a) Ex vivo amplification may be aimed at expanding stem cells or committed progenitors or generating mature blood cells. At least three culture systems are available for in vitro expansion, including stroma-free culture, Dexter-type culture and transwell culture. When CD34+ CD45RAlo cells are cultured in a stroma-free, serum-containing system for 7-21 days in the presence of SCF, IL-3, IL-6, Epo a large increase (3 to 4 logs) in the total cell number and a 8-25-fold increase in progenitor cells is observed, which is associated with the maintenance of the more primitive LTC-IC. (b) Future approaches might also include treatment of stem cells with agents designed to inhibit the action of chimeric genes by blocking gene expression or protein function or manipulating cell signalling pathways with inhibitors of protein-tyrosine kinases. In conclusion, immunological, physical, biochemical strategies of stem cell selection and purification may soon become the method of choice in hematopoietic transplantation.

003

GRAFT VERSUS HOST DISEASE

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The pathogenesis of graft versus host disease (GvHD) is still uncertain, 30 year after it was first described. The animal model has clearly shown that GvHD, in its acute form, is seen when mature donor lymphocytes are infused in an immunocompromized host. The severity of the disease is dependent on 3 variables at least:
1. the degree of immunoincompetence of the host.; 2. the degree of disparity within the major histocompatibility complex (MHC) and 3. the number of infused donor lymphocytes. The experience with allogeneic marrow transplantation (BMT) has confirmed that this is the case also in humans.

HLA matched donor recipient pairs. This is very clear when donor and recipient are mismatched at the MHC locus. It is much less clear when this disparity does not exist, as with HLA (human leukocytes antigen) identical sibling pairs. In fact if we consider 100 HLA matched donor recipients pairs, receiving a T cell replete marrow graft, approximately 50% will not develop acute GvHD, 25% will develop aGvHD which can still respond to immunosuppressive treatment, and 25% will develop life threatening aGvHD. This is what we have observed during the past 20 years in the presence of post-graft immunosuppression with methotrexate (MTX), cyclosporine (CyA) or both. Some pilot studies would suggest that, in the absence of post-BMT is, 100% of the patients will develop severe acute GvHD. Whether it is 25% or 100%, it is quite clear that lethal aGvHD can develop in spite of genetic identity of the HLA locus.

Is aGvHD genetically determined? This would suggest in turn that there must be an other locus relevant for graft/host interactions, but we have failed so far to identify it. Actually there are arguments against aGvHD being genetically determined: 1. GvHD is seen with increasing intensity as the donor's age increases; 2. GvHD is associated with viral infections (such as CMV); 3. some patients receiving a graft from HLA mismatched donors at one locus survive the transplant.; 4. aGvHD will be more severe with very intensive conditioning regimens. The uncertainty on the genetic disparity capable of inducing a T cell response is seen when functional tests are set up to predict GvHD.

Predictive tests. One would expect aGvHD to be the in vivo counterpart of a secondary mixed lymphocyte reaction (MLR): T cells are activated after interaction with alloantigens - a cytotoxic T cell response is mounted - cytotoxic CD8+ T cells kill the target. Unfortunately we have been unable to reproduce these events in vitro between HLA genetically identical pairs: it is very difficult to raise cytotoxicity in this setting and also proliferation. Cytotoxic T cell precursors (CTLPs>) have been raised in HLA phenotypically identical unrelated pairs, but not in genotypically identical family pairs.

Helper T cell precursors (HTLPs) have been claimed to predict aGvHD in HLA identical grafts. This has not been confirmed by other groups including ours: the test is rather cumbersome, it implies generation of EBV transformed B cell lines from the recipient, secondary MLR with donor responder cells against the EBV transformed target and cloning of IL2 producing T cells: the read-out of the test is the number of IL2 producing clones and the quantity of produced IL2. Some labs have claimed a predictive effect of mixed-skin lymphocyte reaction, under the assumption that the skin should contain antigen presenting cells and antigens capable of producing in vitro GvHD. Again the test is cumbersome and the read out on skin histology is far from being reproducible. At present we can not tell if a patient, transplanted with unmanipulated marrow from his HLA identical brother, will develop aGvHD or not.

We can use some of the described risk factors, but nothing more: thus aGvHD will be more frequent in older patients, in CMV serum-positive patients, in ABO minor mismatched combinations, in patients receiving very intensive conditioning regimens.

GvHD prophylaxis. The standard in vivo prophylaxis includes CyA and MTX: this combination was first used by Ray Powles in mismatched grafts, and was then employed by the Seattle group in the matched situation. We have shown that low dose CyA (1 mg/Kg) is less toxic and better tolerated than high dose (5 mg/Kg). We are now exploring the association of low dose CyA with low dose MTX: in patients with acute non lymphoid leukemia (ANLL) in first remission this combination will produce an actuarial 2 year transplant related mortality (TRM) of 4% which is slightly better than the 10% TRM for low dose CyA alone.

T cell depletion abrogate GvHD altogether, but is unfortunately associated with three serious problems: 1. severe and prolonged immunodeficiency, leading to lethal infections; 2. increased risk of graft rejection and 3. increased risk of relapse.

Thus survival has been overall unchanged or worse when compared to unmanipulated marrow grafts.

Treatment of GvHD. Treatment of aGvHD is based on steroids: pilot studies using antilymphocyte globulin (ALG), T cell monoclonal antibodies, anti-IL2 receptor monoclonals, anti-TNF antibodies and FK506 have all given results superimposable with what is seen with steroids. The Italian group GITMO is running a randomized trial testing 10 mg/kg vs 2 mg/kg given very early within 48 hours from diagnosis: hopefully this trial will say whether early treatment and early intensive treatment is better than *chase therapy*, tailoring dose and timing of steroids to the severity of the disease. The latter approach is often unsuccessful because the tissue damage has already been caused, and it is then useless to increase immunosuppression in a patient who is already immunoincompetent, with disrupted barriers, and exposed to opportunistic infections.

Chronic GvHD is an entity on its own: it occurs conventionally beyond day 100 and resembles closely autoimmune diseases: scleroderma like lesions and miositis being the most common manifestations. It is now recognized that chronic GvHD should be treated intensively with one, two or three drug combinations, the 3 drugs being cyclosporin, steroids and azathioprine. Treatment should be continued for many months and often many years, but results may be very rewarding. In refractory cases, or in patients who do not tolerate long lasting cyclosporine, encouraging results have been obtained with PUVA (psolaren + ultraviolet rays) especially on skin lesions.

004

EXPRESSION OF P-GLYCOPROTEIN IN NORMAL TISSUES AND TUMORS

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A variety of analytical methods have been successfully used to detect P-glycoprotein (P-gp) in normal tissues and tumor samples. The currently available data identifies three distinct types of human tissues which express P-gp: subsets of epithelial cells, endothelial cells of capillary beds in specific anatomic locations and placental throphoblasts. By both specific antibodies (Ab) and gene probes, high expressions of P-gp have been found in kidney, liver, colon and adrenal glands. Anatomically P-gp is concentrated on the lumenal surface of cells with a polarized secretory function as the biliary canalicular pole of hepatocytes, the brush border of proximal renal tubular and the apical surface of columnar epithelial cells in the small and large intestine. This pattern of the expression of epithelial cells suggests that P-gp acts as a drug transport protein with an excreting and detoxifying function. A low expression was found in stomach, lung, ovary, uterus, spleen, skin, heart, skeletal muscle and central nervous system. The expression of P-gp in the capillaries of the brain, endoneurium, testis and papillary dermis of the skin, but not midsize and large blood vessels is associated with the continuous non fenestrated arrangement of endothelial cells of these sites, and the recognition of these anatomic locations as blood tissue barriers. Low P-gp levels are found in early and late myeloid precursors, in peripheral blood and bone marrow lymphocytes. P-gp is highly expressed in normal hematopoietic progenitors (CD34+). Our own data shows that the amount of P-gp in lymphocytes, monocytes and granulocytes, as expressed by the degree of reactivity to MRK-16 monoclonal Ab, was always small and varied from undetectable levels to levels comparable with those of non-MDR tumor cell lines. Several patterns of P-gp expression in solid tumors can be identified from the present data. Group 1 tumors, arising from tissues that normally express MDR1, such as renal, colon, adrenocortical and hepatocellular carcinoma have high MDR1 expression levels. Clinically these tumors are all intrinsically drug resistant and have a very low response rate to first-line chemotherapy (CHT). Group 2 includes neuroblastomas, soft tissue sarcomas and breast carcinomas, deriving from tissue with minimal or no mdr1 expression, that occasionally have high-intermediate P-gp levels at presentation, but it could be significantly expressed in relapse following CHT. Group 3 tumors, like ovarian, lung and bladder cancer have almost always undetectable P-gp expression levels. Concerning hematologic malignancies, our data in acute leukemias suggests, according to recent literature, that P-gp overexpression is more frequent at relapse than at first diagnosis and that failures were more frequent in P-gp overexpressing cases. Moreover in acute myeloid leukemia several independent groups showed a relation between P-gp/MDR1 expression and treatment outcome. In lymphomas too, the detection of P-gp positive neoplastic cells at presentation seems to be of clinical relevance and in our group of pts it appeared to represent an independent prognostic factor.

005

P-170 INHIBITION

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P-170 glycoprotein wide expression is not only linked to detoxifying functions of cells but is appears to be also related to their differentiation stage and/or their activation.
Besides attempts to inhibit P-170 activity by drugs able to compete for the binding of P-170 to anticancer agents or by interfering with protein kinase activities, it seems possible to modulate the expression of the protein itself.
Interesting results using antisense oligonucleotides have been reported but a definitive inhibition of P-170 expression has not been reached yet.
As P-170 appears to be inversely related to differentiation in hematopoietic cells, we evaluated the role of G-CSF in the HL-60 cell line. G-CSF was able to reduce the expression of mdr1 mRNA even if the protein was not detectable by immunocytochemical studies.
Recently P-170 expession was induced in a non neoplastic, MDR negative human cell line by cotransfection of the oncogenes c-Ha-ras and c-erbB-2. As neither ras nor erb were able by itself to induce P-170 expression we attempted to inhibit its expression by switching off erb gene in cotransfected cells. This was possible because the gene activity is related to the metallothioneins promoter that is activated by CdCl2 and it is possible to grow the line with or without this latter agent.
All together these results suggest that it is possible to inhibit P-170 expression by acting on the differentiation or the activation of MDR positive cells.

006

MDR REVERSAL AGENTS IN VITRO

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It is known that multidrug resistance is a very important problem in the treatment of cancer. A major goal in experimental and clinical investigation is to discover methods by which to reverse or circumvent it. Through the identification of drugs that antagonize MDR, it is possible to understand better various biochemical mechanisms involved in this form of drug resistance and to provide agents to use in clinical trials. Since 1981 several substances have been found to conteract *in vitro* p170 function. These substances are all believed to be sustrates for the p170 and include calcium channel blockers, calmodulin inihibitors, hormonal derivatives, antibiotics, cardiovascolar drugs, cyclosporines. One of the most important problems in clinical use of chemosensitizer agents is the high dosage needed to neutralize the p170 and its consequent toxicity. We investigated the effect *in vitro* of clinically achievable doses of Cyclosporine A, of its non-immunosuppressive derivative PSC 833 (Sandoz) and of the D-isomer of Verapamil (Knoll) on the toxicity (MTT test) and on the intracellular uptake (flow cytometric assay) of daunorubicin (DNR) and idarubicin (IDA), in the CEM cell line system and in blast cells from 52 patients with acute leukemia. Using the MTT assay in the resistant cell line (CEM VLB) low doses of CyA (1.6 μM) or of D-Ver (3 μM) alone could not abolish p170 mediated resistance of DNR, but a resistance index close to 1 (i.e. a complete inihibition of P170) was obtained by using PSC 833 alone (0.8-1.6 μM) or a combination of CyA and D-Ver. For IDA, a RI close to 1 was obtained with all three modifiers alone or in combination. Analogous results were obtained considering the intracellular content: in the resistant line PSC 833 alone or combinations of CyA and D-Ver increased the intracellular DNR at the level of the parental line. All three modifiers completely restore the cellular uptake of IDA. In blastic cells expressing p170 (mean MRK MFI=7,8±3,1) all the three modifiers significantly increased intracellular content of DNR and IDA ($p = 0{,}000001$). For both antrhacyclines the maximum effect was obtained with a combination of PSC 833 (1.6 μM)+D-Ver 3 μM ($p=0.00000$). This data confirms that low doses of MDR modifiers are able to interact with p170 function not only in experimental systems but also in wild *blast cells*. Clinical trials are warranted useful in confirming their activity *in vivo*, and in better defining their tolerabily and toxicity.

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007

MDR REVERSAL AGENTS IN VIVO

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In the past few years several studies have demonstrated that intrinsic or acquired overexpression of p170 protein negative influences response and survival in a number of hematological and non hematological malignancies (*Chan et al, N. Engl J Med 1991; Pirker et al, J Natl Cancer Inst, 1992; Marie et al, Blood 1991; Campos et al, Blood 1992; Michieli et al, Eur J Hematol, 1992*). This evidence and encouraging results both *in vitro*, and in animal models, provided a strong rationale for the clinical use of MDR modulators. Various lypophilic chemosensitizers including calcium channel blockers, calmodulin inhibitors, cyclosporine and its derivatives, hormones, have been tested in pilot clinical trials, but the results of most of these studies were disappointing: high response rates were achieved in lymphomas, and multiple myelomas, whereas in solid tumors only a few patients appeared to benefit, and in all these cases there were many serius side-effects.
Several reasons could explain the predominantly negative therapeutic outcome; the small number of patients included in the studies, sometimes even with different malignancies; the use of modulators that could not achieve *in vivo* the optimal concentration required for reversal of resistance *in vitro* because of cardiac or other toxicities, or because of alteration of reversal agents bioavailability by serum proteins; heterogeneus expression of p170 within a population of tumor cells; presence of different mechanisms of resistance (topoisomerase II or GST). The effective therapeutic role of MDR modifiers remain to be defined in more carefully designed trials, possibly using new reversal agents, with high potency and less toxicity (i.e. the cyclosporine derivative PSC 833) or using combinations of chemosensitizers with synergistic activity but with non overlapping toxicities (i.e. D-verapamil + cyclosporine). Moreover, the study should include an accurate monitoring of p170 expression, using comparable standardized methods. Finally, pharmacokinetic studies are needed not only to assure the adequate concentration of cytotoxic drug and modulating agents but to evaluate the changes on cytotoxic drug pharmacokinetics and pharmacodynamics due to the block of the p170 function. This information should be helpful in preventing an enhancement of toxicity in normally overexpressing tissues.

008

MULTIDRUG RESISTANCE RELATED TO THE ALTERATED EXPRESSION OF TOPOISOMERASE II (TOPO II) AND ANIONIC GLUTATHIONE-S-TRANSFERASE (GSTπ).

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Drug resistance, whether *de novo* or acquired, represents one of the major obstacles to effective cancer chemotherapy. Among the mechanisms which are thought to contribute to the so-called *atypical* MDR the decreased catalytic activity of Topo II and GSTπ gene overexpression have to be considered. Topo II is an essential nuclear enzyme that catalyzes the interconversion of topological form of double-stranded DNA; in doing so it plays an important physiological role in the process related to cell growth and division. Recently, there has been considerable interest in Topo II as the target of certain classes of antineoplastic agents including anthracyclines, epipodophillotoxins, amsacrine and mitoxantrone. These antitumor drugs interact with Topo II and form drug-enzyme-DNA ternary complexes which trigger a number of biochemical reactions leading to cellular death. Thus, the presence of a sufficent amount of Topo II in cells is predicted to be a prerequisite for drug action, but a mutation of the Topo II gene that affects one of the steps in the Topo II-DNA reaction can also theoretically induce a decrease in Topo II's catalytic activity. A very low Topo II content in cancer cells may constitute the basis of a new mechanism of drug resistance operating in human malignancies, with a large compartment of non proliferating cells, like CLL. Some evidence referring to patients with solid tumors suggests that a low Topo II expression could be a useful marker for clinical response to adriamycin. The glutathione-S-transferases (GSTs), anionic (π), basic (α), and neutral (μ) are a family of detoxification enzymes involved in the metabolism of a broad range of compounds and play an important role in the protection of cells from carcinogens and cytotoxic drugs including alkylating agents, nitrosureas, mitoxantrone and adriamycin. High levels of GSTπ have been associated with the emergence of cell lines resistant to alkylating agents or adriamicyn and, *in vivo*, increased GSTπ gene expression has been found in leukemias, lymphomas and many human solid tumors respect to the matched normal tissues. Although much has been learned about the *typical* multidrug resistance associated with overexpression of P170-glycoprotein, we are just beginning to understand the significance of multidrug resistance associated with alterated Topo II and GSTπ. Increasing evidence suggests that drug-resistance phenomenon may be sustained by more than one mechanism by which tumor cells acquire the ability to survive in presence of lethal doses of cytotoxic agents. According to this hypothesis it has recently been shown that two or more different mechanisms of drug resistance can be expressed at the same time especially in those leukemias and lymphomas with a clinically unfavourable course.

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009

MECHANISM OF RETINOIC ACID RESISTANCE IN ACUTE PROMYELOCYTIC LEUKEMIA

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All-trans retinoic acid (T-RA) is very effective in inducing complete remission in acute promyelocytic leukemia (APL) patients: 84% overall and 95% in patients in whom t(15;17) was documented by cytogenetic or molecular analysis. The response rate is also high in patients resistant to chemotherapy, whereas results are less favorable in second or subsequent relapse. However, the duration of the hematological remission after T-RA is generally brief. Few patients are still in CR one year post-T-RA therapy; mean duration reported in different studies varies from 1 month to 2 years. Despite the fact that de novo resistance to T-RA is very rare, relapsing patients are invariably resistant to further treatment with T-RA.

The cellular mechanism of T-RA sensitivity *in vivo* seems to be the terminal differentiation of the APL blasts. Although the molecular mechanism awaits formal proof, there is experimental evidence that PML/RARα underlies the sensitivity of the promyelocytic blasts to T-RA. Indeed, there is a strict correlation between the expression of the PML/RARα fusion transcript and response to treatment. In addition, PML/RARα increases the sensitivity to RA *in vitro*; and clones of the APL NB4 cell line become T-RA-resistant when they lose PML/RARα expression.

Resistance to RA that develops post-therapy at the time of relapse can be partially explained on a pharmacokinetic basis. As treatment proceeds, the bioavailability of the drug is lowered by an as yet unidentified mechanism. However, in most cases of post-RA therapy relapse, the promyelocytic blasts are resistant *in vitro* to the differentiation action of RA, which suggests that during treatment they have accumulated mutations in molecules involved in the response to RA. Candidate molecules are those dedicated to the intracellular transport of RA (cellular-retinoic-acid-binding proteins, CRABP) and those involved in the response to RA (PML/RARα, RARα, RXR). RA-resistant subclones of the NB4 cell line were shown to have lost the expression-of the PML/RARα protein, suggesting that an alteration of the fusion protein is associated to retinoic acid resistance.

010

GENES ASSOCIATED WITH NEOPLASTIC TRANSFORMATION

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Cell growth is regulated by a balance between proliferation, growth arrest and eventually programmed cell death (apoptosis). The first molecular studies on oncogenesis have focused on the regulation of cell proliferation with the definition of oncogene as the altered version of a gene (proto-oncogene) normally involved in the positive control of cell proliferation. The mutations which turn on a proto-oncogene in an active oncogene are dominant and of the gain of function type. Subsequently, a new category of tumor-associated genes, has been identified as related to growth arrest or negative control of cell proliferation and some of them molecularly cloned. The analysis of tumor suppressor genes has indicated their recessive mode of action and, consequently, the loss of function nature of their mutations.

Oncogenes and tumor suppressor genes define the molecular basis of the multi-step model of neoplastic transformation and provide novel markers for a molecular approach to tumor diagnosis and prognosis. In this context, relevant examples are provided by the formation of oncogenic fusion transcripts following chromosomal rearrangements juxtaposing two different genes, in the case of several hematologic malignancies and the mutations of the tumor suppressor gene p53 identified in most of the solid tumors. The latter genetic alteration, has been characterized both as an early event related to tumor onset and as a marker of tumor progression, depending on the tumor type considered.

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011

STRUCTURAL ALTERATIONS OF THE NF-KB TRANSCRIPTION FACTOR NFKB2/LYT-10 IN LYMPHOID MALIGNANCIES

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Several types of human lymphoid malignancies are associated with chromosomal translocations involving the immunoglobulin or T cell receptor loci and a variety of protooncogene loci. Best understood examples are the t(8;14), t(8;22) and t(2;8) translocations and c-myc gene involvement; the t(14;18) translocation and bcl-2 gene involvement; the t(11;14) translocation and bcl-1 gene involvement. Among others, chromosomal alterations affecting band 10q24 are recurrently associated with lymphoid malignancies, suggesting that this genomic region may contain a proto-oncogene locus. In this context, we have recently identified a novel putative proto-oncogene, a member of the NFkB family, the NFKB2/lyt-10 gene, involved in a t(10;14)(q24;q32) chromosomal translocation occurring in a case of B-NHL, juxtaposing it to the IgH Ca1 locus and leading to a transcriptionally active lyt-10-Ca1 fusion gene. To better characterize the structure and functions of lyt-10 and its role in lymphomagenesis, we have determined the genomic organization of the lyt-10 gene and we analyzed the frequency of rearrangements involving this gene in a large panel of lymphoid malignancies. The lyt-10 gene is composed of 24 exons spanning about 8 kb of genome. The first two exons are non-coding, exons 3-24 contain coding sequences, while the translation termination site, 3' untranslated sequence and the polyadenylation signal are in exon 24. We have investigated the frequency of rearrangements of the lyt-10 locus in a panel of 258 cases representative of different types of lymphoid malignancies by Southern blot hybridization to genomic DNA probes representative of 5' and 3' regions of the lyt-10 locus. Rearrangements involving the lyt-10 locus were found in seven cases: one case of B-cell chronic lymphocytic leukemia (B-CLL), two cases of cutaneous B-cell lymphomas (CBCL), two cases of MM, and two cases of cutaneous T-cell lymphoma (CTCL). The analysis presented here show that rearrangements of the lyt-10 gene are found in different types of lymphoid neoplasia, including B-cell lymphoma, multiple myeloma and T-cell lymphoma indicating that lyt-10 is involved in B-cell as well as in T-cell tumors. The pattern of lyt-10 gene rearrangements in cases reported here indicate a complete or partial deletion of the ankyrin domain. Although alterations of lyt-10 gene may occur by other mechanisms, including point mutations or small internal insertion/deletions, it is conceivable that the loss of the ankyrin domain by genomic rearrangement may represent a general mechanism of constitutive activation of lyt-10 gene *in vivo*.

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012

TUMOR SUPPRESSOR LOCI IN B-CELL NON-HODGKIN LYMPHOMA: PATHOGENETIC ROLE AND CLINICAL IMPLICATIONS

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The role of tumor suppressor loci in the pathogenesis of B-cell non-Hodgkin lymphoma (B-NHL) is well established in the case of the p53 gene and of deletions of the long arm of chromosome 6, whereas other tumor suppressor genes are known not to be involved despite frquent B-NHL deletions at their chromosomal sites (e.g. RB1) or have not yet been investigated in detail (e.g. APC, DCC, p16, WT-1, NF-1). The involvement of p53 in B-NHL development has been documented by extensive studies (1-6), which have shown that: 1) among B-NHL arising *de novo* in the general population, p53 inactivation is restricted to Burkitt lymphoma (BL; 30% of cases). In BL, p53 mutations occur independently of the tumor geographical origin (Europe and North America or Equatorial Africa and New Guinea) and of clonal tumor infection by Epstein Barr virus. The pathogenetic role of p53 inactivation in BL is documented by the growth arrest observed after transfection of a wild type p53 gene in BL cell lines carrying p53 mutations; 2) p53 inactivation is a virtually constant genetic lesion associated with the histologic transformation of follicular B-NHL (BCL-2+; p53–) to diffuse B-NHL (BCL-2+; p53+). It is of note that de novo diffuse lymphomas, which are consistently p53 normal and carry frequent BCL-6 rearrangements, display a distinct genetic pattern from transformed diffuse lymphomas. 3) p53 mutations are a frequent event in AIDS-related small non cleaved cell lymphoma (60% of the cases), whereas they are never detected in other AIDS-related NHL subtypes, i.e. diffuse large cell B-NHL or anaplastic B-NHL; 4) p53 mutations occur in 10% of B-cell chronic lymphocytic lekemias, independently of Rai's clinical stage or lymphocyte doubling time; conversely, p53 inactivation is frequently occurring in Richter's syndromes and represents the only known genetic lesion in this disease. 6q deletions, occurring in 25% of the cases, represent the only other tumor suppressor locus which has been characterized at the molecular level in B-NHL (7). Two regions of minimal deletion have been identified, defined as RMD-1, comprising 1-2 Mb within 6q27, and RMD-2, mapping to 6q21-6q23. Deletions of RMD-1 are classicaly associated with follicular and intermediate grade B-NHL, whereas high grade B-NHL tend to display the RMD-2 type of deletion. In large B-NHL series, 6q deletions have been shown to be a poor prognostic marker.

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013

EXPRESSION OF p53 PROTEIN IN ACUTE MYELOID LEUKEMIA: CYTOFLUORIMETRIC ANALYSIS

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Expression of p53 protein was investigated by flow cytometry in blast cells from 73 patients with acute myeloblastic leukemia at diagnosis. Two different monoclonal antibodies (MoAbs) were employed: Pab 1801 reacting with an epitope present in every form of p53 (wild-type and mutant p53 protein) and Pab 240 which binds only denatured wild-type and an epitope of mutant p53 protein. In blast from 27/73 patients p53 was not detected by any MoAb, while in 46/73 cases (63%) p53 protein was found. In 12 of them p53 protein was identified by both MoAbs, but in the remaining 34 cases blast cells were positive only with Pab 240. No correlation between p53 protein expression and probability to achieve a complete remission was observed. In 11 cases cytofluorimetric expression of p53 protein was assessed at diagnosis and at relapse. At diagnosis p53 was found in 1/11 cases, while at relapse 5 additional cases became positive. Molecular analysis of these 11 leukemic samples showed a p53 gene mutation only in 1 case at relapse.

In conclusion, mutant p53 protein assessed by flow cytometry was found in most acute myeloid blast cells mainly with Pab 240 Moab. These results suggest that the detection of mutant p53 protein is principally due to conformational modifications rather than gene mutations. Furthermore, our findings in 11 AML selected patients, studied at diagnosis and relapse, do confirm the association of altered p53 and progression of the neoplastic event.

014

p53 GENE ALTERATIONS IN THE ACUTE PHASE OF MYELOPROLIFERATIVE DISORDERS

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Chronic myeloproliferative disorders are a heterogenous group of hematogical malignancies due to the clonal expansion of a multipotent stem cell. In these conditions, after a frequent indolent course, a terminal phase characterized by the presence of a deeply altered blastic population with the characteristic of acute leukemia may occur. The most studied disease belonging to this group is Philadelphia-positive chronic myelogenous leukemia (Ph+ CML) that is characterized by the presence of the chimeric protein p210 derived from the fusion of the c-abl proto-oncogene with sequences of the bcr gene. P210 plays a clear role in CML chronic phase. The molecular pathogenesis of the bcr-abl negative myeloproliferative disorders is still largely unclear. The existence in the chronic phase of these disorders of a neoplastic hit controlled, in some way, by a negative regulator which can be abolished, giving rise to the expansion of a blastic clone, is an attractive hypotesis. Therefore alterations of the tumor suppressor gene P53 are potential candidates to play such a role in the progression of myeloproliferative syndromes.

We have studied the DNAs extracted from peripheral leukocytes of 27 Ph+ CML blast crisis and of 8 agnogenic myeloid metaplasia (AMM) acute phase by Southern blot finding an abnormal restriction pattern of the P53 gene in 3 CML and 2 AMM cases. In order to search further subtle structural alterations of the P53 gene, 27 CML cases have been studied by single strand conformation polymorphism (SSCP) analysis of the amplified products representative of the coding region of the gene from exon 4 to exon 9. In 4 CML blast crisis cases an altered SSCP pattern was present. Point mutations have been confirmed and defined by direct sequencing of the same amplified segments. Moreover in one case it has been possible to find the same point mutation (a G to T transversion) present at the same time on exon 7 and exon 8 of the gene. The mutations observed were, therefore, 5 in 4 cases. It is interesting to note that we found point mutations in all the 3 cases of extramedullary blast crisis present in our study.

These results indicate that alterations of the P53 gene are present in a significative number of cases examined and may have a definitive, although not prevalent, role in the progression of myeloproliferative disorders.

015

INDUCTION OF MULTIDRUG RESISTANCE (MDR) BY TRANSFECTION OF MCF-10A CELL LINE WITH C-HA-RAS AND C-ERB-B2 ONCOGENES

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Proto-oncogenes are implicated in cellular proliferation and differentiation and mutations involving them can lead to the development of human cancers. Moreover it has been reported the appearance of resistance to some anticancer drug after oncogene transfection. To better investigate the relationship between oncogene activation and emergence of multidrug resistance (MDR) (a phenomenon related to the P-glycoprotein overexpression on the cell membrane and responsible for cell-resistance to several unrelated anticancer drugs) we transfected the human breast epithelial cell line MCF-10A, negative for the expression of the P-glycoprotein, with c-Ha-ras and/or c-erbB-2 oncogenes. The appearance of the MDR phenotype was then studied by evaluating the mdr-1 gene expression, the presence of the P-glycoprotein on the cell membrane and the sensitivity to the anticancer drug doxorubicin. Briefly, total RNA from MCF-10A (parental and transfected) was subjected to RT/PCR Southern blot analysis. A MDR specific amplification product was detectable only in MCF-7/Dx (positive control) and in MCF-10A co-transfected with c-Ha-ras and c-erbB-2 (MCF-10A HE). These results were confirmed by the immunocytochemical detection of the P-glycoprotein with the monoclonal antibody JSB-1. Only MCF-10A HE exhibited an intense immunostaining along the cell membrane. The expression of the MDR phenotype was further analyzed by evaluating the cellular sensitivity to doxorubicin. This revealed the appearance of drug resistance (reversed by the addition of verapamil) only in MCF-10A HE.

As it has been shown that the co-transfection has an additive effect on the *in vitro* transformation of MCF-10A, we believe that the MDR expression, in this cell line, could be related to the increased malignant transformation.

016

THE BIOLOGICAL AND TRANSACTIVATING ACTIVITIES OF THE ACUTE PROMYELOCYTIC LEUKEMIA PML/RARα PROTEIN DEPEND ON THE FUSION OF THE PML AND RARα COMPONENTS

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Acute promyelocytic leukemia (APL) is characterized by a 15;17 chromosome translocation with breakpoints within the retinoic acid a receptor (RAR) gene on 17 and the PML gene, that encodes a putative transcription factor, on 15. A PML/RAR (PR) fusion protein is formed as a consequence of the 15;17 chromosome translocation. We have shown that PR expression in myeloid cells: 1) blocks vitamin D induced myeloid differentiation; 2) increases cell sensitivity to RA; 3) reduces apoptotic cell death by serum (U937 cells) or GM-CSF (TF-1 cells) deprivation. This effect was potentiated by RA; 4) riduces erythroid differentiation, spontaneous or hemin induced (K562 cells).
These biological activities of PR recapitulate critical features of the APL phenotype. Expression of the PR protein in fibroblasts induced cell death, suggesting a tissue-specific interference of PR with the molecular pathways of apoptosis.
We have studied the mechanism of action of the PR protein with two approaches: 1) we expressed the PML and RAR proteins as well as a number of PR mutants deficient of the PML or RAR DNA binding and dimerization domains in myeloid and fibroblast cell lines. The analysis of cell phenotypes indicate that integrity of both the PML and RAR DNA binding and dimerization domains is required for the whole spectrum of the biological effects of the PR protein. Similar results were obtained by co-expression of the PML and RAR portions of the fusion protein, suggesting that the fusion is required for the PR biological activity. 2) Based on the hypothesis that PR acts as a bifunctional transcription factor, we studied the regulation of known genes in APL blasts and in U937 myeloid cells that express the fusion protein.
We found that PR represses the expression of the adhesion receptor ICAM-1 in the absence of RA, whereas strongly induces ICAM-1 expression in the presence of RA. This effect may contribute to the development of the retinoic acid syndrome that frequently occur in RA-treated APL patients. We also found down-regulation of c-myc expression by PR in U937 cells. This effect was strongly potentiated by RA. The down-regulation was due to reduced trancript initiation. Transient contransfection experiments showed that the c-myc promoter is differentially regulated by PR in different cell lines and c-myc regulation depends on the integrity of all functional domains of the fusion protein. Myc regulation might be part of the mechanisms by which PR controls cell survival.

017

RELEVANCE OF THE PML/RAR-α REARRANGEMENT IN THE DIAGNOSIS AND CLINICAL MANAGEMENT OF ACUTE PROMYELOCYTIC LEUKEMIA

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Cloning of the RAR-α and PML genes involved in the t(15;17) abnormality has provided on the one hand relevant new insights in the pathogenesis of APL. In addition, this discovery has had a tremendous impact in the clinical setting. In fact:
1) The PML/RAR-α hybrid mRNA resulting from the translocation is detectable by RT-PCR using specific primers. This method allows a rapid diagnosis (6-7 hours from sample collection to PCR results). In light of: a) the specificity and consistency of this genetic lesion; b) the frequent association of life-threatening coagulopathy; and c) the specific response to differentiating agents, prompt identification of APL is mandatory for immediate treatment. With respect to therapy with all-trans retinoic acid (ATRA), which is now always included with conventional chemotherapy as a first option treatment, it is important to observe that absence of the PML/RAR-α hybrid in some rare cases classified as FAB M3 has been associated with unresponsiveness to ATRA. For example, the recently described variant t(11;17) aberration which involves RAR-α and a new transcription factor named PLZF, belongs to this category. 2) Preliminary results of minimal residual (MRD) evaluation by PCR have shown that patients with PML/RAR-α transcripts detected in remission after consolidation therapy undergo relapse of their disease within 6-8 months. In contrast, long-term survival is associated in APL with PCR negative tests, thus suggesting that PCR negativity could be considered as the best terapeutic goal in these patients. However, a few relapse have also been observed in patients with no detectable PML/RAR-α cells at remission, probably due to sub-optimal sensitivity of the assay. Based on these findings, the Italian cooperative groups GIMEMA/AIEOP have designed a new clinical study for the treatment of APL (AIDA protocol), which includes PCR evaluation at remission and distinct post-consolidation approaches according to molecular data.

018

REARRANGEMENTS OF THE ALL-1 GENE ON CHROMOSOME 11q23 IN HUMAN ACUTE LEUKEMIAS

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The chromosome band 11q23 has been shown to be frequently involved in translocations and other genetic aberrations found in acute leukemias (AL). Interestingly, several chromosome partners may participate in recombinations with 11q23 band, making this region the most promiscuous genetic site known to be involved in DNA rearrangements of leukemia. We and others have recently isolated and characterized a new gene, named ALL-1 (or Htrx, Hrx, MLL), which is rearranged in AL patients showing 11q23 abnormalities. Several lines of evidence suggest that the ALL-1 gene may be involved in early steps of human development and differentiation. In fact: 1) ALL-1 encodes a putative transcription factor showing A-T hooks, zinc-finger motifs and other regions typically found in several known transcription factors; 2) ALL-1 shares sequence homology with the Drosophila tri-thorax gene involved in the control of embryonic development and differentiation; 3) Alterations of this gene in AL are most frequently (but not exclusively) observed in infants aged less than 1 year and probably result in these cases from a genetic damage occurred during fetal life; 4) Finally, the involvement of ALL-1 in AL showing early maturation arrest with both myeloid and lymphoid features, suggests that the rearrangements may occur in an as yet uncommitted precursor and that the gene should play a role in the proper differentiation of hematopoietic cells. Although the ALL-1 locus spans 100 Kb, all breakpoints are restricted to a restricted 8.3 Kb region, and can be easily detected on Southern blot using a cDNA probe and a single enzymatic digestion. Given to the formation of hybrid fusion protein in several 11q23 recombinations (e.g. t4;11 and t9;11), an RT-PCR approach has been developed to identify this abnormality at diagnosis and to monitoring residual disease. Clinically, ALL-1 gene rearrangements are usually associated with an extremely poor prognostic outcome, particularly in infants. In this latter clinical subset, the ALL-1 gene status is an independent prognostic factor which may be used in the future to better address treatment strategies.

019

HETEROGENEOUS TRANSCRIPTS INDUCED BY THE t(1;19) IN ACUTE LYMPHOBLASTIC LEUKEMIA

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The t(1;19) (q23;p13), occurring in the leukemic cells of 6.5% of children with ALL has been shown to produce a hybrid E2A-PBX1 gene that is expressed in a typical set of oncogenic chimeric proteins. In the majority of pre-B cell ALL harbouring the t(1;19), a consistent joining site in the chimeric E2A-pbx1 transcripts has been detected. Nevertheless, more recent observations suggested that cytogenetically identical 1;199 translocations can be heterogeneous at a molecular level, resulting in different species of fusion messengers.
In an attempt to identify these molecular variants, we screened 25 pediatric cases of pre-B cell ALL both by Southern blot, to detect E2A gene rearrangementes, and by RT-PCRF, to detect the chimeric E2A-pbx1 transcripts.
In addition to seven cases with the molecular pattern usually associated with the t(1;19), we identified three molecular variants.
One case showed a variant E2A-pbx1 transcript that consists of 27 additional base pairs inserted in frame at the joining site and is identical to that recently reported by Izraeli et al. In two cases Southern blot evidenced the expected E2A gene rearrangement, but extensive RT-PCR analysis failed to detect any E2A-pbx1 transcript. Since the cytogenetic analysis confirmed the presence of the translocation, it can be suggested that a gene other than PBX1 might be involved in these t(1;19) variants. To check for this hypothesis, we are setting up RACE experiments using E2A gene specific primers as an anchor to isolate possible new fusion transcripts.
The isolation of the t(1;19) variants could provide both some insights in the molecular mechanisms leading to this translocation and a more powerful test for its molecular detection.

020

BCR/ABL PROTEINS AND DIFFERENT LEUKEMIC PHENOTYPES

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The presence of a Ph– chromosome in human leukemias parallels the presence of molecular defects involving the Abl oncogene which is translocated to chromosome 22 and fused with sequences of the Bcr gene. As the breakpoint positions within the Bcr gene may vary, different chimeric genes, in which the Bcr coding sequences are represented ina variable extent may result. In chronic phase CML, breakpoints occur almost invariably in the so-called *mbcr* region and two different types of chimeric mRNAs are present which differ for the presence of the *mbcr* exon 3 sequences (75 bp) and codify for two slightly different P210 proteins. A third type of Bcr/Abl junction has been reported in Ph'-positive acute leukemias; In approximately 50% of these cases, as a consequence of a breakpoint position occurring within the first large intron of the Bcr gene, the Bcr first exon sequences are linked directly to Abl exon 2. The resulting Bcr/Abl abnormal protein expressed is 190 Kd in MW (190) and has been so far detected mainly in cases showing an acute phenotype, although its presence has been described also in sporadic cases in chronic phase. We have identified the existence of a fourth type of Bcr/Abl junction, which has been so far identified in few CML patients showing an unusual breakpoint very 3' within the Bcr gene. In these patients, the Northern analysis reveal the presence of a Bcr/Abl message of approximately 9.5 Kb and therefore longer than that normally expressed in CML. All the patients carrying this abnormal type of junction are patients showing a *benign* clinical and hematological CML phenotype. Taken together these data suggest that the length of the Bcr portion in the Bcr/Abl fusion proteins may influence the *in vivo* biological activity of the hybrid proteins and this, in turn, may be associated with the appearance of different leukemic phenotypes and different prognosis of the associated diseases.

021

THE C-ABELSON PROTO-ONCOGENE: AN OVERVIEW ON ITS STRUCTURE AND FUNCTION

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The product of the c-ABL protooncogene belongs to the non-receptor class of tyrosine protein kinases (TPKs). Like all members of this group, the c-ABL possess an SH1 domain (the *catalytic* domain) which is the specific site of the enzymatic activity. The SH2 and SH3 domains are located aminoterminal to the catalytic domain; the SH2 site is capable of high affinity binding to specific phosphotyrosin containing proteins and it is believed to play a critical role in the interaction between signaling components of the TPKs' dependent pathways. SH3 sequence recognizes a proline rich ligand (3BP1) known to be involved in the regulation of activity of small G proteins like Ras, Rho and Rac.
Recently, the COOH- terminal portion of c-ABL molecule was found to contain a domain involved in binding to F-Actin as well as a separate domain with DNA binding properties.
Furthermore, the c-ABL protein phosphorylates the mammalian RNA polymerase II and is capable of forming a complex with retinoblastoma protein, providing unequivocal evidence for its partecipation in transcriptional regulation.
Thus, the c-ABL TPK appears to be a multifunctional enzyme with multiple substrates and protein interactions; in addition, because c-ABL contains both F-Actin binding domain and nuclear localization signal, it has been postulated to play a pivotal role in the signal transfer pathway from the cytoskeleton to the nucleus.

022

C-ABL FUNCTION IN THE PROLIFERATION OF CD34+ NORMAL HUMAN HEMOPOIETIC PROGENITORS

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The c-abl proto-oncogene encodes for a protein with tyrosine kinase activity. It has been shown that the suppression of c-abl expression results in the selective inhibition of normal human colony forming unit-granulocyte macrophage (CFU-GM) growth.
Aim of this study was to evaluate the effects of the suppression of c-abl expression on the clonogenic growth and on the cell cycle kinetics of CD34+ normal human hemopoietic progenitor cells. Gene expression was inhibited with 18 base-unmodified antisense oligodeoxinucleotides complementary to the first six codons of the two alternative c-abl first exons, Ia and Ib, corresponding respectively to the 6.0 kb and 7.0 kb c-abl mRNAs. Bone marrow-derived CD34+ cells were obtained by immunomagnetic selection and incubated for 17 hours at 37°C in the presence of the appropriate oligodeoxinucleotides, at a final concentration of 15 mM; at the end of the incubation an aliquot of the cells was assayed for clonogenic growth, the remaining were used for flow cytometric (FCM) analysis. Preincubation with both the antisense oligomers produced a significant inhibition (51%±11; $P<0.01$) of CFU-GM growth, while BFU-E growth was not significantly affected; two sense oligomers had no effect. FCM analysis of both DNA content and bromodeoxyuridine incorporation showed that median S-phase of CD34+ cells was 19.2% (range: 13-25%) in control cultures, 6.8% (range 4-10%) in antisense treated cultures ($P<0.005$) and 15.3% (range 13-20%) in sense treated cultures (P not significant). Preincubation with antisense oligomers resulted in an evident accumulation of cells in the G0/G1 region of the DNA histogram without any evidence of apoptosis. Absence of significant apoptosis was confirmed also by agarose gel electrophoresis of DNA.
These results suggest that suppression of c-abl expression by antisense oligodeoxynucleotides inhibits the S-phase progression of CD34+ human marrow cells, without inducing apoptosis, and that inhibition of clonogenic growth of the human hemopoietic progenitors is probably produced by this effect. Therefore, c-abl function seems to be important for entry of human hemopoietic progenitors into the S-phase of the cell cycle.

023

STRUCTURAL ORGANIZATION OF BCR GENE IN CHRONIC MYELOID LEUKEMIA (CML) PATIENTS AND ITS ROLE ON BCR-ABL NEOPLASTIC ACTIVATION

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The bcr gene encodes a phosphoprotein associated with a serine/threonine kinase activity. In particular, the first bcr exon contains autophosphorylation activity and transphosphorylation activity. These functions may be the molecular basis of the activation of bcr-abl oncogene, by direct interaction between bcr sequences and the tyrosine kinase regulatory domains of abl. The binding domains has been localized within the first exon of bcr and consists of at least two SH2 domains.
Recent data suggest that bcr sequences in bcr-abl could functionally substitute for myristillation and deletion of the SH3 domain in activating the abl oncogene. We studied 102 DNA samples of CML patients for the presence of mutations in the first exon of the bcr gene, dividing the DNA region into four amplified fragments by polymerase chain reaction(PCR).
By single strand conformation polymorphism analysis (SSCP) and direct sequencing of amplified fragments, we found different banding profiles in 36 out of 102 patients in the PCR fragment spanning nucleotide 506-826. In one patient, sequence analysis revealed the presence of a point mutation at nucleotide 669. In the others mutated fragments we found a polymorphism, not associated with CML or other phase of the disease.
Our results demonstrated the presence of point mutation in this regulatory region, and may suggest a role for the altered bcr sequences in activation of the bcr-abl oncogene.

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024

BCR/ABL: STRUCTURE AND FUNCTION

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As a consequence of the t(9;22), the Abelson (ABL) protooncogene, normally located on chromosome 9, moves to a very restricted area of chromosome 22, spanning for only 5.8 kb, called *major breakpoint cluster region* (BCR). This region belongs to a gene composed of a large number of exons and introns called BCR gene. The functions of the BCR gene are poorly known.
However, a GTP-ase function has been ascertained. In particular, a large number of functions have been recognized on the first exon, that one conserved in all the types of different BCR/ABL hybrids, independently of the occuring breakpoint. These functions have been described in another part of this session.
On the other side ABL gene has a tyrosine kinase (TK) activity which, in normal conditions is of a consistently low level. It is supposed that this activity is severely inhibited, in normal conditions, by the presence of inhibitors which can have access to the TK site of the p145 ABL protein. The formation of the hybrid inevitably leads to a modification of the three-dimensional protein structure.
According to recent computer-assisted models, the BCR portion of the hybrid covers some parts of the ABL molecule, due to the presence of SH2 hormology motifs in the exon 1 portion of the BCR.
Moreover, the central part of the BCR gene, which comprises exon 2 through exon 12 contains domains structurally homologous to dbl oncogene and cdc 25 gene, which encodes for a cyclin. This part of the BCR gene is present in both the p210 variants of CML, but is lost in the p190 protein. The presence or absence of these domains could explain a different behaviour of the two proteins on different substrates or on different cell lines.

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025

PERSISTENCE OF NON CLONAL HEMATOPOIETIC PROGENITOR CELLS IN BLASTIC PHASE CHRONIC MYELOGENOUS LEUKEMIA (CML)

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Normal and clonal hematopoietic progenitor cells have been demonstrated to coexist in chronic-phase chronic myelogenous leukemia (CML) but few data are available on the presence of non neoplastic hematopoiesis during the blastic transformation phase.
We investigated by reverse transcription-polymerase chain reaction (RT-PCR) for expression of the BCR/ABL transcript of individual hematopoietic progenitors from a CML patient in blastic phase.
We demonstrate that non clonal hematopoiesis is induced to reemerge by conventional chemotherapy including fludarabine.
In addition, we confirm that some pluripotent CD34+/CD33–/DR– cells circulating in the peripheral blood are not clonal.
Our data provide an encouraging basis for further studies addressing the issue of the *in vitro* purification of normal hematopoietic stem cells in advanced stages CML and their use in the setting of autologous bone marrow transplantation.

026

EFFECT OF BCR-ABL ANTISENSE OLIGONUCLEOTIDES ON CELLS FROM PATIENTS WITH CML IN CHRONIC PHASE: POSSIBILITY TO IMPROVE ANTILEUKEMIC ACTIVITY USING CD34+ CELLS

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The exposure of leukemic basts from patients with CML in blastic crisis to synthetic 18-mer oligonucleotides complementary to the BCR-ABL junction suppresses leukemic colony formation whereas normal granulocyte-macrophage colony formation was uneffected. Similarly, in leukemic mice sistemically treated with a BCR-ABL antisense oligonucleotides, no leukemic cell were detected, nor did leukemia colonies form in semisolid medium. We have investigated whether leukemic mononuclear or CD34+ cells from patients in chronic phase show similar sensitivity to antisense oligonucleotides after incubation *in vitro* with different antisense concentrations and prolonged period of incubation. To concentrate CD34+ cells, CML cells were separated over a 1.077 g/mL density gradient to obtain mononuclear cells and then further layered over a discontinuous Percoll gradient (1.065 g/mL) to enrich in hematopoietic progenitors. The final population of CD34+ concentrated cells was separated using a cocktail of monoclonal antibodies and immunomagnetic beads (negative depletion). Incubations with antisense oligonucleotides were carried out in Iscove's medium supplemented with 4% human AB serum, rec-IL3 and rec-GM-CSF. Treated cultures received 40 ug/mL of 16-mer or 26-mer B3A2 or B2A2 sense or antisense at the start of the culture period; the same or a 50% reduced dose was added to the medium after 24, 48, and 72 hours. An aliquot of non-adherent cells was plated in methylcellulose at time 0 and after 24 and 72 hours. Using the 26-mer oligonucleotides, a significant major antileukemic effect was observed using higher oligonucleotides concentrations, effect not confirmed when the 16-mer oligos were used. In addition, with the longer antisense we have been able to show a significant time-dependent effect (24-hrs vs 72-hrs) and a specificity of antisense as compared to the sense and to the junction-not related antisense. When CD34+ cells were evaluated, an antileukemic effect similar to the mononuclear cells was found; however, the specific effect of antisense improved, both with the 26-mer and the 16-mer oligonucleotides. Evaluation of oligonucleotides uptake by mononuclear cells during the incubation time showed a progressive accumulation of oligonucleotides into the cells, suggesting that re-addition of antisense to the medium might increase Ph-positive cell elimination.

027

PHILADELPHIA CHROMOSOME FUSION PEPTIDES AS A POSSIBLE TARGET FOR A CD8+ T CELL RESPONSE

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The brc/abl fusion gene on Philadelphia chromosome codes for a protein, p210, that is responsible for malignant transformation in CML. Theorically, non-self peptides originating from the junction region could be presented as nonamers by HLA class I molecules and induce a CD8+ T cell response. Two different junction sequences (b3-a2 or b2-a2) can be found in p210, depending on which bcr exons are maintained at the N-terminus, possibly leading to the formation of nine b2-a3 and nine different b3-a2 nonapeptides. It is not known whether these peptides can bind to HLA class I molecules to be presented to the patient's lymphocytes and, if yes, to which allelic molecules. To answer to this question a panel of 18 synthetic nonapeptides that overlap by eight residues and span the b2-a2 and b3-a2 junction sequence of the p210 fusion protein were tested for binding to HLA class I a chains, using the a chain refolding assay set up by Dr. Tanigaki. HLA molecules were purified from 15 HLA homozygous B lymphoid cell lines, covering seven A-locus (A1, 2, 3, 11, 24, 31, 32) and nine B-locus (B), 18, 27, 35, 37, 44, 51, 54, 62) alleles. Collectively the results indicate that: 1) Ten Ph' junction peptides show HLA class I binding; 2) most of the peptides which bind are derived from the b3-a2 junction sequence; 3) for two b3-a2 peptides the class I allele involved in the binding can be identified as HLA-A3. Therefore, patients carrying both the b3-a2 fusion and the HLA-A3 molecule could be potential candidates for a specific immunotherapy. If the presence of HLA-A3 is able to naturally protect against CML it should be significantly less frequent among b3-a2 than among b2-a2 patients. From the comparison of HLA-A and B phenotype frequencies in 150 patients characterized for the bcr/abl rearrangement no significant difference was identified. This could mean that none of the peptides is generated or presented *in vivo*. It could anyhow be that they are presented in a non-efficient way and that an immune response can be enhanced *in vitro* by stimulating the patient's lymphocytes with the synthetic peptide capable to bind to his HLA class I molecules.

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028

ALTERATION OF CELLULAR HOMEOSTASIS INDUCED BY IBRID GENE BCR/ABL

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The *chronic myeloid leukemia* (CML)-associated oncoprotein p210 has pleiotropic effects involving multiple pathways of cellular regulation. The proliferative advantage of CML clone is maintained by p210 bcr/abl which, as other classes of tyrosine kinase oncogenes, constitutively phosphorylates and activates cytoplasmic proteins involved in growth factor-mediated signal transduction or altenatively creates an autocrine loop.
Clonal expansion of CML hematopoiesis results from either disregulated cell proliferation and prolongation of cell survival. p210 bcr/abl impaires cell surface cytoadhesion molecule expression: abnormal attachment of CML most primitive progenitors to the stromal compartment of hematopoiesis could underlie lack of responsiveness to microenvironment-derived control factors, inappropriate release from bone marrow and extramedullary hematopoiesis.
Moreover, p210 bcr/abl perturbs cell cycle distribution and inactivates signals that block cells at the G1/S boundary.
Finally, p210 bcr/abl promotes cell survival, by inhibiting apoptotic cell death. Both altered cell cycle progression and prolonged cell survival may play a critical role in increasing cell susceptibility to secondary genetic events and transformation to blast crisis.

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029

ESTABLISHMENT OF NESTED PCR FOR DETECTION OF THE HYBRID BCR/ABL mRNA IN SINGLE PROGENITORS CELLS FROM CHRONIC MYELOID LEUKEMIA PATIENTS

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The Philadelphia (Ph) chromosome, resulting from a reciprocal translocation of chromosome 9 and 22, is the specific marker of chronic phase of CML. The molecular consequence of the Ph chromosome is the formation of a fusion gene bcr/abl wich produce a P-210 protein tyrosine kinase. Several experimental protocols have been proposed to select Ph- hematopoietic progenitor cells for autologous transplantation. The correct evaluation of the benign or malignant nature of purified progenitors requires a highly sensitive, specific and reproducible technique allowing fast detection of the hybrid gene. Polymerase chain reaction (PCR) has provided a sensible method to detect and amplify the hybrid bcr/abl fusion mRNA in heterogeneous marrow non- purified progenitors cells. The aim of the present study was to establish a nested PCR technique for detection of the hybrid bcr/abl mRNA in single progenitors cells. Progenitors cells from the bone marrow of CML patients, normal donors and Ph-positive cell line EBV 173 (mRNA b2a2) were grown in metylcellulosa cultures. Individual 14 day colonies were removed. Total RNA was isolated using a TRIzol™ reagent method (GIBCO BRL). To ensure the isolation of small amounts of RNA we had added as a carrier 4-5 μg of MS2 phage RNA (Boehringer). Complementary DNAs were syntetized using a hexa random primer (5'-ACGTAT-3') and M-MLV reverse transcriptase (GIBCO BRL). PCR amplification was performed using 4.5 mL of cDNA in a finale volume of 45 ml. The mix PCR was constituted by 4.5 μL Taq buffer 10x (100 mM Tris-HCl ph 8.8, 30 mM $(NH_4)_2SO_4$, 1.6 mM $MgCl_2$), 4.5 μL of dNTP 2.5 mM, 26 pmol of each primers and 2 unit of Taq pol. Thirty five cycles of PCR (94°C 20 sec, 60°C 30 sec and 72°C 60 sec) was performed in a thermal cycler (Perkin Elmer 480). The primer sequences are: b1b2: 5'-GAA GAA GTG TTT CAG AAG CTT CTC CC-3' a2a3: 5'-GAC CCG GAG CTT TTC ACC TTT AGT T-3' abl: 5'-TTC AGC GGC CAG TAG CAT CTG ACT T-3'. The primers b1b2 and a2a3 detect the hybrid bcr/abl message while the primers abl and a2a3 detect abl exon 2 (internal positive control). A nested PCR are performed using 2 μL of the first PCR product and the same PCR condition. The sequence of nested primers are: b2: 5'-GTG AAA CTC CAG ACT GCT CAC AGC A-3' a2: 5'-TCC ACT GGC CAC AAA ATC ATC ATA CAGT-3'. In the nested PCR the same internal positive control is used. The reaction products were electrophoretically separated through a 2% agarose gel and stained with ethidium bromide. The expected products generated by PCR were: 378 pb (b3a2) and 303 pb (b2a2) for the first PCR and 272 pb (b3a2) and 197 pb (b2a2) for the nested PCR. By using this thecnique, single colonies derived from bone marrow of normal donors or CML patients as from the Ph-positive cell line EBV 173 were analyzed to validate the approach described herein. More than 95% of the individually removed colonies were successfully analyzed. This tecnique was highly specific detecting the lack of hybrid mRNA in all normal colonies and the presence of hybrid bcr/abl mRNA in colonies generated by bone marrow of CML patients and cell line EBV 173. In conclusion, this technique can be used as a fast, sensible and specific approach to analyse CML progenitor cells.

030

DETECTION OF BCR-ABL TRANSCRIPTS BY PCR IN CML PATIENTS RECEIVING ALLOGENEIC BONE MARROW TRANSPLANTATION

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The prognostic significance of detection of bcr-abl transcripts by PCR in bone marrow samples of patients undergone to allo-BMT for chronic myelogenous leukemia is still not completely ascertained. We evaluated the presence of minimal residual disease in 11 CML patients by nested RT-PCR.
Ten of the 11 pts were transplanted in first chronic phase, one in accelerated phase. Eight patients received HLA matched siblings marrow without T cell depletion and two a MUD allograft. We analysed 43 bone marrow samples at different time intervals (1-36 months) after BMT. Seven of fortythree (16%) samples were PCR positive, but none of the patients had signs of hematological relapse. The bcr-abl splicing pattern was prevalently b3a2 (4/5 cases). PCR positivity reappeared in one case two months after BMT, and remained positive up till 10 months, after which BM samples converted to negativity. In three cases samples converted to PCR positivity 12 months post BMT, among these one MUD allograft, presenting b2a2 as splicing pattern. Another of the three pts converted again to PCR negativity at 24 months post BMT. Moreover, in one patient PCR positivity was revealed 36 months after BMT and at present time (52 months) is still in remission. From our data, even if for a limited number of patients, results that PCR conversion in bone marrow samples can occur independently of disease status at the moment of BMT and even relatively late post BMT, without being followed by hematological relapse. We noted a prevalence of PCR positivity with b3a2 splicing pattern, but in two cases further PCR analysis showed revertion to negativity. Whether such molecular features indicate only modulation of the immunological system of the patients or are indeed heralding with cospicuous anticipation a CML relapse,is a biological problem which to be clarified needs extended follow-up periods.

	cases with convertion PCR+		*cases with convertion PCR–*	*relapse*
	before 12 m	after 12 m		
b3a2	1	3	2	0
b2a2	0	1	0	0

031

IN CELL REVERSE TRANSCRIPTASE POLYMERASE CHAIN REACTION (RT-PCR) FOR THE DETECTION OF HYBRID m-RNA IN CHRONIC MYELOID LEUKEMIA PATIENTS

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Methods aiming to the detection of disease in chronic myeloid leukemia (CML) include chromosome analysis, Southern blotting, polymerase chain reaction (PCR) and *in situ* hybridization techniques (FISH). We describe a method for the detection of the hybrid BCR-ABL transcript within the single cells.
After cellular permeabilization and fixation, the hybrid m-RNA was reverse transcribed in c-DNA and the c-DNA was amplified by PCR, using fluorescent specific primers. The amplified DNA was detected within the cells cytoplasm by fluorescent microscopy and flow cytometry. With this technique, we have studied four Ph+ CML patients and two normal subjects for the control. The first and the second patients (pts) were studied at diagnosis and the karyotypes were 100% Ph positive, while *in cell* RT-PCR showed 293 positive and 4 negative cells in the former case and 195 positive and 8 negative cells in the latter. The third case showed a minor cytogenetic conversion after 8 months of α-IFN treatment: 20 out of 50 metaphases were Ph negative. *In cell* RT-PCR revealed a lower number of negative cells. The fourth case was a patient submitted to BMT. The karyotype, RT-PCR and *in cell* RT-PCR studied about 9 years after BMT were negative. In the two normal subjects *in cell* RT-PCR was negative. Flow cytometry also was used to analyze m-RNA sequences in the cellular cytoplasm.
This method appears equally reliable and cheaper with respect to FISH technique and can be employed in other hematologic malignancies, characterized by translocations leading to the formation of hybrid genes.

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032

DOUBLE COLOUR FISH DETECTION OF BCR/ABL GENES FUSION IN CHRONIC MYELOID LEUKEMIA PATIENTS ON IFN-α THERAPY: A FAST AND POWERFUL TOOL IN CLINICAL LABORATORY

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We investigated the feasibility in a clinical laboratory routine of dual colour FISH analysis, with BCR/ABL genes probes, of Ph-positive cells in chronic myeloid leukemia patients whose cytogenetic analysis after IFN-α therapy gave inconsistent results. In all 30 bone marrow specimens were tested: from CML patients at diagnosis (10 samples) from CML patients after IFN-α treatment (10 samples) and from Ph-negative subjects, used as negative controls (10 samples). Remarkably all 30 specimens, were analyzed easily and successfully, though owing to some technical problems our hybridization procedure had to be modified.
Worth of note the rate of Ph positivity in IFN-α treated patients seemed to differ significantly if investigated by metaphases scoring or by FISH analysis (mean shifting value 34%). Surely this result can be attributed to the low number of evaluable metaphases we found in cytogenetic preparations from IFN treated subjects, but maybe it could also be ascribed to the ability of FISH to score bcr/abl fusion in two different compartments: resting cells and dividing cells.
On the basis of our results we think this method could be of great clinical utility for optimizing treatment and bone marrow harvesting timing in CML patients on interferon therapy. As in Ph negative controls 1 up to 4% of positive fusion signals were observed this approach, so far, seems not to be suited for minimal residual disease detection.

033

IMMUNOPHENOTYPIC APPROACH TO THE DETECTION OF MINIMAL RESIDUAL DISEASE IN ACUTE LEUKEMIA

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The prerequisite for using immunophenotyping for detecting morphologically unrecognisable residual neoplastic cells (minimal residual disease: MRD) during complete remission (CR) in AML and ALL is that leukemic cells express antigenic patterns which are not present on normal bone marrow (BM) cells. A single antigen can not be used to distinguish neoplastic from lympho-hematopoietic cells, since the same antigens can be found in both cell populations. Therefore, the *leukemia-specific* antigenic patterns to be used as markers for the detection of MRD are always represented by the co-expression by the same cells of two (or three) antigens (Ags) which in a given patient have been previously demonstrated to characterise all blasts at presentation.
Such combinations, which include membrane as well as cytoplasmic (e.g. CyCD3) and/or nuclear Ags (e.g. TdT), can reveal, using appropriate cytofluorimetric techniques, one leukemic cell in 104 normal BM cells. Overall, the detection of MRD by *leukemia-specific* markers can be applied to: >95% of T-ALL [CyCD3-CD5/TdT], 20-25% of B-lineage ALL [CD13-CD33/CD19, CD56/CD19], 30% of AML [CD34/CD56, CD13-CD33-CDw65/TdT, CD13-CD33-CDw65/CD7]. The possibility of phenotypic alterations during treatment is a potential weakness of immunophenotypic detection of MRD. However, modifications of the antigenic profile of leukemic cells occur rather rarely.
On the basis of available data, the immunophenotypic approach to the detection of MRD in acute leukemia, although not universally applicable, appears as a reliable additional tool for the surveillance of the CR. Preliminary studies strongly suggest that the surveillance of the MRD by immunophenotypic methods is of clinical usefulness in predicting the occurrence of leukemia relapse.

034

IMMUNOLOGICAL APPROACH TO MINIMAL RESIDUAL DISEASE

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Many methods were studied to obtain a more exact definition of complete remission, among these the immunological method is the easiest to obtain in laboratory involved in leukemia diagnosis. A great number of studies have been conducted to evaluate the efficiency of immunologic methods to identify leukemic cells. A consensus of which method should be used has not been achieved by now.
We studied normal and leukemic cells using the double fluorescence in cytofluorimetry to evaluate which leukemic phenotype most frequently has a counterpart in non leukemic bone marrow.
To increase the efficiency of our test we considered a number of cells between 100,000 and 250,000 using 4 different gates with 5 biparametric and 2 monoparametric histograms.
This method resulted very efficient identifying 5-10 out of 250,000 cells. In 50% of leukemias the applied method was efficient and a study of minimal residual disease with surface antigens was possible. An analogue method has been used in myeloid leukemia, a pathology which presents even higher difficulties in morphologic interpretation. Our results detected the presence of atypic lymphoid markers (most frequently CD4,CD7, and CD19) in over 70% of myeloid leukemia which allowed subsequent monitoration of minimal residual disease.
Our study shows that the immunological approach is a simple and easy method to identifies leukemic cells in low concentration and might result helpful in the evaluation of complete remission.

035

MINIMAL RESIDUAL DISEASE ANALYSIS IN ACUTE MYELOGENOUS LEUKEMIA

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Molecular cloning of the genes interrupted by the chromosomal translocations specifically associated with subtypes of acute myelogenous leukemia (AML) has recently disclosed the possibility of evaluating the presence of minimal residual disease (MRD) by the RT-PCR technique.
About 20-40% of AML-M2 are associated with the t(8;21) translocation; the breakpoints are located in the AML1 gene on chromosome 21 and in the ETO gene on chromosome 8. A chimeric AML1-ETO gene is actively transcribed and translated into a fusion protein. As the junction point in the fusion cDNA is identical in all analysed cases, a specific amplification method has allowed the detection of chimeric AML1-ETO transcript.
AML-M4Eo is known to be associated with inv(16)(p13q22), which is characterized at the molecular level by a fusion transcript involving the 5'part of the CBF-β gene on 16q and the smooth muscle myosin heavy chain gene (MYH11) on 16p. RT-PCR analysis has identified 4 breakpoints in the MYH11 gene, a single one occurring in > 80% of the cases.
The t(6;9) translocation is found with AML characterized by poor prognosis, basophilia in bone marrow and young age. The fusion gene DEK-CAN codes for a hybrid protein capable of DNA binding, acting as a transciption factor.
All these cytogenetic aberrations are therefore subject to PCR analysis. The clinical value of the RT-PCR technique in the follow-up of leukemic patients has been shown in AML-M3, where the finding of a hybrid PML-RAR gene predicts relapse. Only a small number of long surviving patients with t(8;21) AML-M2 has been analyzed up to now: in all cases PCR showed the presence of cells expressing a AML1-ETO transcript during hematologic remission. Persistence of residual cells carrying the chromosomal translocation in long-term disease-free patients indicates that clinical relevance of PCR positivity may differ in distinct subtypes of leukemia.

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036

DETECTION OF MINIMAL RESIDUAL DISEASE IN PH+ CML PATIENTS AFTER OPPOSITE SEX TRANSPLANTATION USING FISH TECHNIQUE WITH X-CHROMOSOME PROBE

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In order to quantify the minimal residual disease after allogenic bone marrow transplantation we studied with FISH technique 23 Ph+ - LMC female patients who received marrow cells from their brothers, using X-centromeric specific probe. As controls we analyzed 10,000 BM cells fixed in acetic acid/methanol from 4 BM donors. The results of controls showed that, on the average, 0.22% of the interphase cells showed 2 signals (SD = 0.26; M + 2SD = 0.74%).
For each patient we examined from 1000 to 2500 cells. Two of the 23 patients, 9 years post BMT, BCR (with PCR technique) negative, 100% donor blood group have been considered as ideal internal controls. 0.51 and 0.41% of these two patients cells exhibited two signals (M = 0.46; SD = 0.07; M + 2SD = 0.6%).
Therefore we consider that percentages > 1% may indicate the presence of hemopoietic cells of the recipient.
Comparing cytogenetic and FISH analysis, some considerations can be proposed:

1. the significance of presence of 2.5-3% of cells with 2 signals (3 cases) has been confirmed by the finding of at least one Ph+ metaphase, increasing the number of examined mitosis from 20-25 to 50-70.
   In these cases BCR rearrangement investigation by molecular techniques is not required;
2. in patients showing 1 to 2.5% of cells with two signals (3 cases), we could not find any Ph+ metaphase (in these cases BCR rearrangement analysis is required);
3. percentages < 1% of host cells do not exclude the possibility of recipient hematopoietic expansion (2 cases);
4. cytogenetic and FISH relapses (> 25% of host cells) do not necessarely mean the failure of the BMT;
5. FISH is able to provide additional informations to cytogenetic analysis with a rapid technique; FISH is able to indicate when it is necessary looking for BCR rearrangement with molecular techniques but it can not replace them.

037

A NOVEL STRATEGY TO AMPLIFY REARRANGED IMMUNOGLOBULIN HEAVY-CHAIN GENES FOR THE DETECTION OF MINIMAL RESIDUAL DISEASE IN B-CELL MALIGNANCIES

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We have developed a novel polymerase chain reaction (PCR) based approach to amplify rearranged immunoglobulin heavy-chain (IgH) genes for detecting minimal residual disease in B-cell malignancies. Antigen specificity in B cells is derived primarily from the rearrangement of IgH variable (V), diversity (D), and joining (J) segments.
Rearranged variable regions (VDJ) contain four framework regions, interrupted by three complementarity-determining regions (CDR) that codify for the antigen-binding site. CDR regions are unique to each B-cell clone, and have been used to define clone-specific primers and probes to detect minimal residual disease. VDJ genes were amplified using one of two sets of consensus sense primers derived from the leader and first framework regions, and a consensus antisense primer derived from the 3' end of the six joining (JH) regions. Amplified VDJs were then directly sequenced or cloned, and the CDR2 and CDR3 identified. A tumor-specific primer was derived from the CDR2 and used with the JH consensus primer to amplify neoplastic VDJ sequences from pathologic samples. PCR products were then hybridized to a tumor-specific probe derived from the CDR3.
The use of a tumor-specific primer and probe gave two steps of specificity to the assay. Our method showed the same sensitivity (10^{-5}) and specificity of a previous method (anchor PCR) in which both the sense and antisense primers were derived from tumor-specific sequences.
We employed this method to analyze a panel of mature B-cell malignancies, including 22 patients with multiple myeloma (MM), 16 non-Hodgkin's lymphoma (NHL) and 8 chronic lymphocytic leukemia (CLL). VDJ region sequences were obtained in 78% of patients (81% of MM, 81% of NHL, and 62% of CLL). It is noteworthy that the same panel of patients was tested using a consensus sense primer derived from the third framework region (*Yamada et al, Proc Natl Acad Sci USA 86:5123, 1989*), showing successful amplifications in only 55% of cases.
We conclude that our assay is sensitive, specific and can be considered a reliable assay for the detection of minimal residual disease in mature B-cell tumors.

038

RAPID POLYMERASE CHAIN REACTION METHOD FOR DETECTING MONOCLONALITY IN B-CELL LYMPHOPROLIFERATIVE DISORDERS

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Conventional diagnosis of lymphoproliferative disorders is typically based on morphology and immunophenotyping. Analysis of the immunoglobulin (Ig) and T-cell receptor gene rearrangements in lymphoproliferative diseases has provided information of practical diagnostic value in distinguishing neoplastic from reactive conditions, assigning cell lineage and determining the extent of the disease. Southern blotting is the standard method for recognizing a monoclonal gene rearrangement, but this techniques is laborious, time consuming, requires radioactive isotopes and a relatively large amount of DNA (10 mg); furthermore it can usually only detect about 5% malignant cells admixed within a polyclonal population. We performed a simple assay to detect the monoclonality in B-cell lymphoproliferative disorders using the polymerase chain reaction (PCR) with two primers at the 3' end of the third framework region (FR3) of the variable region and at the 3' end of the joining segments of the Ig heavy chain gene. Genomic DNAs (1 mg) were amplified for 35 cycles and PCR products were electrophoresed on 3% agarose gels or, in some cases, were analyzed on 10% polyacrylamide gels; the gels were stained with ethidium bromide and viewed on a UV transilluminator. Positive samples were characterized by a 80-160-bp amplification fragment and the polyclonal pattern formed a smear in the same molecular weight range. In all experiments we included samples of genomic DNAs from monoclonal and polyclonal B and T cells as positive and negative controls, respectively. In addition, mixtures without template were included as negative contamination-free controls.
Monoclonality was detected in peripheral blood (PB) or bone marrow samples of 8/10 B-cell acute lymphoblastic leukemia (B-ALL), 11/11 hairy cell leukemia (HCL), 10/10 B-cell chronic lymphocytic leukemia (B-CLL) and in 3/7 B-cell non-Hodgkin's lymphoma (B-NHL), whereas the amplification of PB samples of two patients with B lymphocytosis produced polyclonal smears; all T-cell samples were negative. Limiting dilution experiments showed that a monoclonal band within a polyclonal DNA may be detected at a level of sensitivity of 1%. The sensitivity of this technique is higher than that of Southern blotting; in fact, in 2 HCL and in 1 B-CLL samples analyzed after prolonged treatment, a monoclonal band, not visible by Southern blotting, could be detected by PCR analysis.
On the basis of our experience, we propose the detection of Ig gene rearrangements using this PCR method for a rapid diagnosis and towards monitoring the follow-up of B-cell chronic lymphoproliferative disorders. The sensitivity and reliability of this method need to be further improved in order to extend its potential application to B-ALL and B-NHL.

039

PCR-BASED DETECTION OF RESIDUAL MYELOMA CELLS AFTER HIGH-DOSE CHEMO-RADIOTHERAPY FOLLOWED BY BONE MARROW AND/OR PERIPHERAL BLOOD AUTOGRAFTING

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The use of high-dose (HD) chemo-radiotherapy followed by autologous transplantation in the treatment of multiple myeloma (MM) has raised the issue of the assessment of minimal residual disease with a high degree of sensitivity. This is particularly relevant for those patients who achieve a complete remission according to conventional criteria. Polymerase chain reaction (PCR) based studies have recently demonstrated that tumor cells are detectable in the peripheral blood (PB) of almost all MM patients at diagnosis. Since the reinfusion of malignant cells is a major concern in autografting procedures, we decided to evaluate whether residual myeloma cells were present in both bone marrow (BM) and PB stem cell harvests used to restore hematopoiesis after the myeloablative treatment. We have developed a novel PCR-based strategy to detect residual myeloma cells using clone-specific sequences derived from the rearrangement of immunoglobulin heavy-chain genes. Rearranged variable regions (VDJ) have been amplified using one of two sets of sense primers (from the leader, and first framework region), and a consensus antisense primer derived from the 3' end of the six joining regions. Amplified VDJs have been sequenced, and the second and third complementarity determining regions (CDR2 and CDR3) identified. Tumor-specific oligonucleotides have been generated from CDR sequences. The use of a primer derived from the CDR2 and a probe from the CDR3, gave two steps of specificity to the residual disease detection: a tumor-specific amplification, and hybridization. We have studied 15 MM patients enrolled in a pilot HD chemo-radiotherapy program. Three cycles of dexamethasone 40 mg/sm preceded the HD regimen which involved the sequential use of etoposide 2g/sm, mitoxantrone (escalating doses from 30 to 60 mg/sm), and cyclophosphamide 7g/sm. RhGM-CSF (5 mcg/kg) has been infused after each drug. The myeloablative regimen consisted of melphalan 120 mg/sm, and fractionated total body irradiation (10 Gy). In 12 of 15 (80%) patients, the VDJ sequence was obtained, and patient-specific primers and probes were generated. To date 9 of 12 patients have completed the HD program. We report that residual myeloma cells were detectable in BM and PB at diagnosis, BM and PB cell harvests, and after transplantation in all patients. Our findings indicate that HD chemo-radiotherapy is not able to provide PCR-negative BM and PB stem cell harvests. In addition, the constant presence of residual myeloma cells in PB cell harvests rules out the hypothesis that leukophereses could provide an uncontaminated source of stem cells.

040

MOLECULAR STUDY OF DIFFUSE INTERMEDIATE AND HIGH GRADE NHL TREATED WITH INTENSIFICATION THERAPY

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Immunoglobulin heavy chain (IgH) gene rearrangement occurs at an early stage of B lymphoid development. This process generates a hypervariable sequence known as the complementary determining region III (CDR III) of the IgH chain. Since B non-Hodgkin lymphoma (NHL) results from the clonal expansion of a malignant B lymphoid precursor, its IgH rearrangement can be exploited as specific marker for the neoplastic clone. The prognosis of NHL has improved with the introduction of dose escalation and intensification therapy rescued by peripheral blood stem cells collected by apheresis. In order to evaluate the real impact of this therapeutic approach the accurate detection of neoplastic cells in apheresis samples, and the persistence of minimal residual disease (MRD) appears mandatory. Using a sensitive method identifying CDR III specific sequences with the polymerase chain reaction (PCR), we evaluated 8 cases of high grade B NHL with bone marrow involvement, treated with intensification therapy. Marrow samples were obtained at diagnosis, to establish the specific rearrangement, during and after therapy. Furthermore available apheresis were studied for each patient. Amplification was performed using 75 pmol of each primer, 2 U of Taq polymerase, and 1 μg of genomic DNA. Positive and negative controls were included in each experiment. An aliquot of the reaction was electrophoresed through 12% polyacrylamide and silver stained. At diagnosis a clonospecific rearrangement was observed in each patient. Furthermore in all cases we obtained additional longer bands, probably due to specific amplification of germline fragments. All but 2 apheresis samples were IgH-PCR positive. After peripheral blood stem cell transplantation disease signal was not detectable in few cases and only in the earlier follow-up samples. Interestingly these patients show longer survival. These preliminary data suggests that better disease outcome may be correlated to disease eradication obtained with high dose chemotherapy, confirmed by the PCR negativity in the early apheresis and in the bone marrow obtained in the first months post rescue. However, the second possibility of survival without disease could be related to a very low residual tumor burden.

041

SENSITIVITY OF RT-PCR IN MONITORING OF MINIMAL RESIDUAL DISEASE

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The bcr-ABL reciprocal traslocation present in >90% of chronic myeloid leukemias (CML) has been routinely detected in the past by cytogenetic analysis of Philadelphia chromosome (Ph). The sensitivity of this method is relatively low and cannot be applied to detect minimal residual disease (MRD). Recently RT-PCR has been successfully applied to monitor MRD in different stages of CML. In this work we compared two methods to assess the sensitivity of RT-PCR of bcr-ABL translocation.
Following the procedure originally described by Kawasaki et al. (*Proc. Natl. Acad. Sci. USA 85, 5698-702, 1988*) and modified by Roth et al. (*Blood 74, 882-5, 1989*), the sensitivity was determined by either limiting dilution of Ph-positive K562 cell line with Ph-negative Jurkat cells or by mixing RNA extracted from positive or negative cells. Reverse transcription was performed with random examers followed by PCR with two sets of nested primers.
Results obtained show that after two round of amplifications with nested primers the sensitivity of the test allows the detection of one Ph-positive cell in a sample containing 10^6 Ph-negative cells with no differences among mixture of cells or mixture of RNA. Nevertheless analysis of amplificate products after the first round of amplification with the outer primers shows that mixture of RNAs is 1000-fold more sensitive than mixture of cells and does not allow the quantitation of positive cells. The samples obtained from patients are represented by Ph negative cells with a low number of Ph positive cells; this, together with our data, suggests that limiting dilution of Ph-positive cells with Ph negative cells should be used as positive control of the assays and allow semiquantitative analysis.
The screening of MRD by RT-PCR on a large number of patients will provide a basis for determining the value of MRD in the prognosis and management of the disease. Furthermore, RT-PCR will be of great importance in monitoring the bone marrow explants for autologous transplantation.

042

SHORT TERM STUDY OF CHIMERISM AND BIOLOGICAL CHARACTERISTICS OF HEMOPOIESIS AFTER BONE MARROW TRANSPLANTATION.

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Over the past two decades, bone marrow transplantation (BMT) using genotypically HLA identical sibling donors has been accepted as the most effective therapy for patient with several hematological disorders. Survival after BMT is largely dependent on the stability of engraftment and the severity of acute and chronic graft-versus-host disease. Our aim was to study chimerism early during hematopoietic reconstitution and to characterize the biological behaviour of this phase. The small number of cells available at early time points post BMT precluded studies of early engraftment kinetics by karyotypic analysis. Red cell surface antigens are inadeguate markers after transplantation because of repeated transfusion and the fact of HLA antigens is limited to mismatched situation. To our purpose in this study we used M27 probe. The M27 probe detects the DXS 255 locus on X chromosome, where a variable copy number tandem repeat (UNTR) sequence is mapped; this probe shows multiallelic variation in sequence lenght and a high heterozygosity rate (80-90%). The use of a sensitive to methylation restriction enzyme (HPA II) allows the distinction of inactive from active alleles. Since active and inactive forms of each chromosome will be present in polyclonal population, HPA II will cleave about half of each allele, leading to four bands. In a monoclonal populations, only one of two fragments will be shortened by HPA II, because the same chromosome will be inactive. Thanks to the multiallelic variation in sequence lenght this technique allows the study of the majority of the patient grafted with a sex mismatched sibling donors and of about the half of the recipients grafted with sex-matched donors. Fifteen grafted patients, eligible to our study, were analyzed in the phase of early recovery of hemopoiesis (i.e. leukocyte > 500). DNA was extracted with standard methods from a sample of peripheral blood. After digestion with PST I DNA was transferred to a nylon plus membrane and hybridized with M27 probe. When the donor was a female a further digestion with HPA II in an aliquot of PST I digested DNA was performed to distinguish clonal composition of hemopoiesis. In all the cases, the analysis of engraftment showed the complete disappearance of recipient signal. Further studies on eligible patient revealed a polyclonal pattern of X methylation in the donor signal. Some points should be outlined:

- Southern blotting method has a low sensibility (about 5%) leading to a misleading exclusion of the presence of residual recipient cells;
- a late engraftment may be correlated with a higher risk of relapse;
- a normal clonal engraftment may occur has demonstrate by the experience of hemopoietic reconstitution of subletally irradiated mouse;
- the polyclonal reconstitution may be correlated to a good engraftment and minor risk of relapse;
- this early analysis may be useful in recognizing earlier engraftment failure;
- this is one of the few methods applicable to the majority of transplanted patients.

043

POLYCLONAL HEMATOPOIESIS AT HEMATOLOGICAL REMISSION IN TWO PATIENTS WITH T(8;21) ACUTE MYELOID LEUKEMIA SHOWING PERSISTENCE OF MINIMAL RESIDUAL DISEASE

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We have studied the hematopoietic reconstitution after remission in two female patients affected by M2 acute myeloid leukemia with a t(8;21) translocation.
In spite of the fact that RT-PCR analysis shows the persistence of cells expressing a AML-ETO hybrid transcript in both patients, they are still in clinical and cytogenetic remission after more than 30 months from achieving remission and off therapy.
In order to evaluate the clonal or polyclonal nature of hematopoiesis in these two patients, we took advantage of an established clonal assay based on the methylation status of the human androgen receptor gene (HUMARA). Two oligonucleotide primers corresponding to sequences located on the Humara first exon allow to amplify a 280 bp fragment wich contains a trinucleotide repeat highly polymorphic in human population. The same fragment contains four restriction sites for the enzymes HpaII and HhaI wich are methylation sensitive. Digestion with these enzyme and subsequent amplification of normal female DNA allow to distinguish the methylation status of the maternal and paternal alleles.
As X-chromosome inactivation is a random event occurring in female after hematopoietic stem cells development, random methylation of both alleles is expected in normal polyclonal hemopoiesis, whereas preferential methylation of one allele will be seen in clonal expansions. Skewed pattern of X-chromosome methylation simulating clonal hematopoiesis may however occur also in a percentage of normal females. A pattern compatible with restoration of polyclonal hematopoiesis was seen in our two patients with t (8;21) AML in remission.
These data support the notion that hemopoietic reconstitution after achieving remission from AML with t (8;21) translocation is mainly sustained by polyclonal (possibly normal) hematopoiesis even if minimal residual disease is still detectable by PCR analysis.

044

QUANTIFICATION AND MONITORING OF THE NEOPLASTIC TRANSCRIPT BCR-ABL IN CML PATIENT BY ELECTROPHORESIS CAPILLARY

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We previously reported the validity of assessing the presence of molecular marker bcr-abl in CML patients during the interferon therapy (IFN) or after allogeneic bone marrow transplantation (BMT). We used the RT-PCR of the chimeric transcript for monitoring the out-come of the disease in 77 allogeneic transplanted patients, from 3- to 10 years after the BMT, and in more than 200 patient during the IFN therapy, from 3 to 4 years after the beginning of the immune-therapy.
The aim of the new our project was to asses the utility of the detection of the leukemic specific amplified product (PCR), and its quantification used to monitoring the variation of the neoplastic clone, during the disease progression or remission, by a *quantitative* method and by the separation and quantification on capillary electrophoresis (EC) of PCR products, a new and automated method.
We applied this method on 10 allo-transplanted patient with *high* risk of clinical relapse and in 10 patients during IFN treatment.
We demonstrated the predictable value of the two methods in assessing *molecular* relapse, few months before the cytogenetic and clinical relapse. Using the *in cell-PCR*, an alternative method for quantification of bcr-abl transcript, which correlate the morphology of cell with the expression of the bcr-abl transcript we also find a correlation of the number of neoplastic cells with the chemotherapy and immune-modulating treatment.
Our preliminary data indicate a new quantitative approach to the monitoring of the neoplasm by RT-PCR.

045

FLUDARABINE AND GEMCITABINE EFFECTS ON HUMAN ACUTE MYELOID LEUKEMIA BLAST CELLS: DIRECT COMPARISON OF CYTOTOXICITY AND CELLULAR ARA-C UPTAKE ENHANCEMENT

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Cytosine arabinoside remains the most effective therapeutic agent in adult acute leukemia. Potentiation of production of the active metabolite (ARA-CTP) by deoxycytidinkinase has been recently achieved by pretreatment of leukemic cells with other arabinosylnucleotides. Such potentiating effect has been observed in vivo as well. We wished to compare the effects of fludarabine (F-Ara-A) and gemcitabine (dFdC), the latter a pirimidine analog that has been shown to be extremely active on solid tumors. We thus evaluated ARA-C uptake and retention after both drug pretreatment, cellular proliferation and apoptosis. The experimental design was such that mononuclear cells obtained from AML patients and cells of the myeloid cell line HL 60 were incubated 37 C, 5% CO_2 for 3 hours in the presence of either F-Ara-A or dFdC at equimolar concentrations (10 uM). Cells were then washed thoroughly and exposed in the same culture conditions to Ara-C 1 uM, immediately (day 0), or after 24h of culture in the absence of any drug (day1). Incubation with Ara-C lasted variable time intervals (0-240 min). At the end of all incubations, 3HAra-C cytoplasmatic and nuclear uptake was evaluated, together with the half life of the tritiated drug. In parallel, 3HTdR uptake and cell cycle (by PI incorporation and FACS analysis) distribution of blasts were evaluated. The percentage of apoptotic cells was also determined by cellular DNA content at a FACSscan and by gel electrophoresis, by the presence of the characteristic ladder of endonucleosomic digestion products. Moreover, AML blasts recovered from cultures were plated in semisolid medium to evaluate cloning efficiency after drug treatment. From our data it is evident that at day 0, F-Ara-A pretreatment induced a significative increase ($p<0.01$) in Ara-C uptake in comparison to untreated and dFdC pretreated blast cells. Such effect is accompanied by a significative inhibition of 3HTdR uptake, by a decrease in the percentage and absolute number of S phase cells, an increase in the peak of apoptotic cells and by a significative inhibition in colony formation ($p<0.05$) in cells exposed to the combination of F-Ara-A and Ara-C respect to those incubated with an unique drug. Although no such synergic effect nor any increase in Ara-C uptake was detectable at day 0 for dFdC, the incubation of AML blasts with this drug could induce a significative inhibition in 3HTdR uptake, in S phase cellular fraction ($p<0.01$) and provoke apoptosis. At day 1, the synergic effects of F-Ara-A and Ara-C on cell proliferation and self renewal remained evident, but no increase in Ara-C uptake was detectable in F-Ara-A pretreated cells. On the other hand, blasts exposed to dFdC and then to Ara-C showed after 24h of wash-out of the drug a significative increase in Ara-C uptake respect to controls and F-Ara-A treated cells. The proliferation of cells was significatively and synergistically inhibited by the combination of dFdC and Ara-C, at a higher rate respect to what observed at day 0. Alltogether, our data indicate that dFdC has strong intrinsic cytotoxic properties on AML blasts, accompanied by a "delayed" effect of potentiation on Ara-C activity. These characteristics, together with the absence of important side effects *in vivo* render dFdC a drug possible to consider as alternative to F-Ara-A in the therapy of refractory AMLs.

046

MDR EXPRESSION DURING AVIAN LYMPHOCYTIC DEVELOPMENT

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The expression of MDR gene by tumor cells is considered a significant obstacle to the effective treatment of malignancies by chemotherapeutic agents. The expression of MDR gene has been reported in some bone marrow derived cells CD34 positive/ CD33 negative, in mature T-lymphocytes and in a minor subpopulation of CD5/CD19 positive lymphocytes isolated from normal donors, the normal counterpart of B-CLL cells. In this report we evaluated the expression of mdr1-mRNA in the thymus and in the bursa of Fabricious of chicken embryos from day 12th of embryonic life to the birth. In the birds, maturation of the immune system cells takes place separately in the thymus for T-lymphocytes and in the bursa for B-cells; so, we can follow the appearance/disappearance of mdr1-mRNA during the lymphocytic system development. Total RNA from tissues was diluted to 300 ng/µl and subjected to reverse transcription to generate cDNA; then, 35 cycles of PCR were performed with upstream and downstream primers chosen in order to amplify one of the two ATP-binding sites, a higly conserved region of mdr1-gene, stable in several species. PCR products were electrophoresed in a 5% acrylamide gel, blotted into a nylon membrane and hybridized with the specific digoxigenin-labeled probe PCH1 (kindly supplied by Pr. Ling) in order to test the specificity of the 450 bp band detected on the gel. Our results indicate that the specific band was present or absent depending upon the age of the embryo and the organ analysed. In the thymus, a clear signal was present from the first day of analysis (day 12th) to the birth. By contrast, the message was absent in the bursa until day 14th and then was present up to the day 17th. After this time, the message disappeared. Taking account the cronological maturative events described in the primary lymphatic organs of chicken, it could be hypothesized that the early lymphopoietic stem cells may express mdr gene(s) during their maturative steps in the primary organs. The CFU-cells populate the primary lymphatic organs by day 7th of embryonic life for the thymus and by day 9th for the bursa. At day 12th begins the Ig genes rearrangement and at day 13th the Ig expression. Between day 14th to 18th there is a continous migration of stem cells into the bursa, with proliferation and then progressive maturation.; from day 18th, when the 85% of bursal cells are sIgM positive, mature B-lymphocytes leave the bursa towards secondary lymphatic organs. Thus, cells mdr-mRNA expressing could correspond to maturing elements above described, the normal counterpart of CLL B-cells. In the thymus, the lymphocytic maturation begins earlier than in the bursa; so, the MDR positive cells here observed could be the mature T-cells, according to other studies that demonstrate the MDR positivity in mature T-lymphocytes.

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dGT SEQUENCES, SPECIFICALLY AND SELECTIVELY INHIBITING GROWTH OF HUMAN CANCER CELL LINES, RECOGNIZE SINGLE-STRANDED DNA BINDING PROTEINS

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The main aim of the cancer research is to identify mechanisms to selectively control cancer cell growth, without interfering with normal cell functions. DNA and RNA protein interactions are some of the molecular processes necessary for cell survival. In particular, in many organisms including higher eukaryotes, single-stranded DNA binding proteins (SSBs) may play an important role in transcription, replication, recombination and repair of DNA, by binding the intermediate single stranded products. Despite the large number of SSBs so far identified, the precise biological function of many of them is yet unknown.
We have identified dGT oligomeric sequences able to exert a specific dose-dependent cytostatic/cytotoxic effect on lymphoblastic CEM and monocytic U937 human cancer cell lines. After 72h of culture, one dose of 15 mM of dGTs caused a 73% and 90% of growth inhibition in CCRF-CEM and U937, respectively. Moreover, after one week of culture, no survival of CCRF-CEM cells may be found. The same results were obtained utilizing the multidrug-resistant VLB-CEM cell line. On the contrary, no toxic effects were found administering to cells oligomeric control sequences of the same lenght, but with different base composition with respect to dGTs. Surprisingly, we found that administration of dGTs to normal human lymphocytes, either resting or PHA-activated, did not alter cell viability or growth, also if they efficiently took up oligomers. Experiments performed administering to CEM lines oligomers by transfection procedure did not result in a change of toxicity, suggesting intracellular oligomer interactions. In gel mobility shift assays, nuclear extracts derived from the cell lines showed to nteract with dGTs giving almost three major bands susceptible to protease digestion. In competition experiments these bands were not displaced by non toxic oligomers and by dGT duplex, indicating that dGTs specifically recognized SSBs. The same experiments performed with nuclear extracts derived from PHA-activated lymphocytes failed to demonstrate dGTs-SSB interactions, whereas control oligomers were able to shift different bands.
These results might prove the existence of SSBs, the function of which could be essential for the viability and growth of some cancer cells. This might open new perspectives in a *SSB-sequestration strategy* to selectively control tumor cell progression.

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DETECTION OF SMALL PERCENTAGES OF MDR POSITIVE CELLS: COMPARISON OF DIFFERENT TECHNIQUES

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Multidrug resistance (MDR) is one of the most important mechanisms involved in the resistance to chemotherapy and also a low number of MDR positive cells can play an important role in therapy failure.
In this study we investigated the capability of some methods used for the study of the P170 glycoprotein in detecting small percentages of MDR positive cells.
For this purpose we used the T lymphoblastic cell line CCRF CEM (CEM) and its resistant subline CEM VLB 300 (CEM 300), mixed together in different proportions. These mixed solutions were tested with different techniques: flow cytometry for the study of P170 expression, the cytotoxic assay MTT and the flow cytometric analysis of drug intracellular content for the P170 functional study.
The expression of P170 was studied by flow cytometry (FACScan, BD) using the monoclonal antibody MRK16. This technique enabled to detect the two different cellular populations even at very low concentrations (1% CEM 300+99% CEM).
For the flow cytometric study of drug intracellular content, cells were incubated for two hours with 1000 ng/mL of daunorubicin (DNR) with or without a reversal agent, SDZ PSC 833 (PSC), used at the concentration of 1.6 μM. Also with this technique we were able to detect the different DNR contents of the two cell lines at very low concentrations (1% CEM 300+99% CEM). With PSC 1.6 μM the CEM 300 DNR intracellular content became as high as CEM one.
For the MTT cytotoxic assay cells were incubated for two days with increasing DNR concentrations. This technique was less accurate in detecting a low number of MDR positive cells. In fact the dose-response curve for the solution 5% CEM 300+95% CEM practically overlapped the curve for 100% CEM.
In conclusion in the study of multidrug resistance it is very important to choose a technique enabling the detection of small percentages of MDR positive cells, which can determine therapy failure.

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CYTOFLUORIMETRIC DETERMINATION OF P-GLYCOPROTEIN IN AML BY A SOFTWARE PROTOCOL BASED ON HISTOGRAM CORRECTION AND CUMULATIVE SUBTRACTION

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Overexpression of P-glycoprotein (PGP) has been identified as an underlying mechanism of multidrug resistance (MDR) and appears to be closely related to clinical resistance in newly diagnosed and relapsed acute myeloid leukemia (AML) patients. PGP expression was found to correlate with lower complete remission (CR) rates and shorter overall survival and remission duration in AML (*Campos et al.,1992; Michieli et al.,1992; Sato et al., 1990; Zhou et al., 1992*). We studied 146 patients with newly diagnosed AML, 69 females and 77 males, median age 56 yrs (range 17-81) diagnosed between January, 1990 and December, 1993. They were treated by intensive chemotherapy including an anthracycline, etoposide, and high doses of cytosine arabinoside. Marrow aspirates were routinely stained and evaluated according to the revised FAB criteria. Immunophenotypic studies were carried out by flow-cytometry with a wide panel of directly conjugated monoclonal antibodies (MoAbs) including CD13, CD33, CD34, HLA-DR, CD7, CD2, CD10, CD19, CD15, CD14. PGP expression was analyzed using the fluorescein isothyocyanate (FITC) conjugated C219 MoAb (CIS Diagnostici, Vercelli, Italy). As the MoAb recognizes an epitope on the inner surface of the cytoplasmic membrane, the blast cells were permeabilized in 3.5% paraphormaldeyde/PBS and, after two washings in PBS, in 50% cold acetone/PBS. Samples were then incubated at 4°C for 30 min with 10 l of FITC-conjugated C219 MoAb. Analysis was carried out by flow cytometry (Epics Profile, Coulter, FL, USA). Given the heterogeneous expression of PGP in terms of number of cells stained or fluorescence intensity, in accord with Campos et al., the threshold of positivity was set to a conventional 20%. In leukemic blasts, the differences in fluorescence intensity between control sample and test sample were often small, resulting in nondisjunct distributions. Therefore, an improved histogram analysis (IMMUNO-4, Coulter, FL, USA), based either on cumulative subtraction or normalization of the control histogram to best match the test histogram, was applied to distinguish antigen-positive from antigen-negative cells. Sixty-six samples (45.2%) were considered positive for P-170 expression. No relationship was found between sex, age, high leucocyte count, organomegaly and the PGP phenotype. Positive correlation was found with the surface marker CD7 ($P< 0.001$), but not with FAB subtypes, CD34 and/or HLA-DR antigens. With regard to clinical responsiveness, PGP+AML patients showed lower complete remission (CR) rates ($P<0.001$) and shorter overall survival ($P=0.001$) compared to the PGP negative ones. The predictive value of PGP on the achievement of CR was confirmed in multivariate analysis. Our study contributes to the identification of patients with poor prognosis, but also suggests that therapeutic trials with P-170 blockers may be important, mainly for patients with MDR-1 positive blast cells.

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REDUCTION OF RHODAMINE-123 EFFLUX BY MDR MODULATORS AND DETECTION OF MDR1 GENE EXPRESSION IN ACUTE LEUKEMIA: PROGNOSTIC IMPLICATIONS

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Expression of the multidrug resistance gene MDR1 is reported to be determinant for response to therapy and survival in some tumors and in leukemia. Several studies have been performed measuring either expression of MDR1 mRNA or its product, the P-glycoprotein. In our study we investigated the MDR expression by a functional flow cytometric assay based on the fluorescent dye Rhodamine-123 (Rhd123) efflux/retention in presence or absence of MDR modulators. The CEM parental cell line and the derived Vinblastine-100 resistant cells (CEM/VLB100) were used as negative and positive controls. Rhd efflux was not seen in the parental cell line whereas in the CEM/VBL resistant it was markedly present (> 1 log reduction of fluorescence intensity) and efficiently blocked by verapamil (10 ug/mL), cyclosporin A (5 ug/mL) and its analog PSC 833 (SANDOZ) (5 uM), respectively by 45 %, 48.9%, and 54.2%. Rt-PCR detection of mRNA for MDR1 was used to confirme the MDR expression in the CEM/VBL cell line. We then studied 92 patients with acute leukemia, 66 of them were at diagnosis and subsequently treated with at least one MDR-related chemoterapeutic agent, the mean blast percentage was > 80%, 60 cases were AML and 32 ALL. Among the AML patients 33% failed to achieve complete remission and a significant ($p=0.005$) difference in the mean Rhd efflux percentage was observed in presence of MDR modulator compared with CR patients (30.1% vs 11.3%). Among the group of non responding cases 78.5% of them showed a significant higher value of Rhd efflux compared to 36% of responding patients. When Rt-PCR was performed the expression of MDR1 was found in 62.5% of the cases and a correlation with the functional test was found in 87.5% of resistant cases. In patients with ALL we did not found difference in terms of efflux of fluorescent dye and MDR1 gene expression between responding and non responding cases, rather in these patients could be important to establish correlation between MDR expression and response duration.

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MULTIDRUG RESISTANCE (MDR) REVERSING ACTIVITY AND PHARMACOKINETICS OF VERAPAMIL, CYCLOSPORIN A, AND R-TELUDIPINE (GR66234A) IN MDR LEUKEMIA CELLS

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Recent studies have shown that there are at least two different drug-binding sites on P-glycoprotein (Pgp): one for verapamil (VER), cyclosporin A (CyA), and vinca alkaloids, and a second for azidopine, a dihydropyridine (DPD) compound. We compared the daunorubicin (DNR) resistance reversing activity of VER and CyA with a new DPD derivative called R-teludipine (GR66234A) that shows a low calcium antagonistic activity and a high lipophilia. MDR cells lines K562, MELC-DRTL, and CCRF-CEM VBL were used in this study. Cells were incubated with 5uM VER, CyA, and GR66234A separately. After three washings to remove each MDR reversing agent, aliquotes of cells were exposed 1h to 2ug/mL DNR immediately after (T0) or 4(T4), 8(T8), and 24(T24) hours after resuspention in drug free medium. Furthermore, samples of cells exposed simultaneously with DNR and MDR reversing agents for 1h were studied. GR66234A results more active than CyA and VER to increase the cytotoxic activity of DNR. The uptake of DNR, evaluated by flow cytometry, in presence of CyA and GR66234A is similar and greater than that observed using VER. Intracellular DNR accumulation and distribution data, evaluated by fluorescence microscope, confirm the results obtained with flow cytometry. When the resistant cells are treated with DNR after removal of each MDR reversing agent, only GR66234A still shows a good MDR reversing activity also in the sample of cells treated with DNR at T24. These data suggest that the bond between GR66234A and Pgp is more stable than that of CyA or VER with Pgp. This can have important effects for clinical use because the sensitization of MDR neoplastic cells may still be present after many hours after administration of GR66234A.

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P-170 EXPRESSION IN UNTREATED MULTIPLE MYELOMA

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Fifty-one consecutive patients with previously untreated multiple myeloma (MM) were investigated for P-170 expression that has been related to multidrug resistance (MDR). They were 26 females and 25 males (median age: 63.5 years; range 40-82). Nineteen patients (37%) had stage I MM, 8 (16%) stage II and 24 (47%) stage III. The M-component resulted IgG in 35 cases (68%), IgA in 10 cases (20%), while 6 cases (12%) were light chain MM.
Bone marrow aspirates were analyzed by an immunocytochemical APAAP technique, using the MoAb MRK-16 recognizing an external epitope of P-170. Strongly P-170 positive bone marrow plasmacells (from 1 to 100% of total plasmacells) were detected in 17/51 (33%). Such a strong P-170 positivity was detected in 6/7 cases (86%) where serum β2 microglobulin (β2M) concentration was higher than 6 mg/dL, and in 10/40 (25%) cases where β2M was lower than 6 mg/dL ($p = 0.007$).
Twenty-two MM cases (16 P-170 negative and 6 P-170 positive) were subsequently treated with MDR-related drugs. Fourteen of the 16 P-170 negative cases (87%) responded to this therapy (> 50% in 13 cases; > 25% in 1 case), while the remaining 2 were resistant. Of the 6 P-170 positive cases, 3 were responsive (> 50% in all) and 3 were resistant to treatment ($p = 0.1$).
In conclusion a rather high portion of previously untreated MM cases may show strong P-170 positivity in bone marrow plasmacells. This appears to be related to increased β2M serum levels. Further studies will clarify whether this might imply a worse response to subsequent treatment with mdr-related drugs.

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EXPRESSION OF GST AND P-170 GLYCOPROTEIN IN MUTLIPLE MYELOMA

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Bone marrow samples from 52 patients affected by multiple myeloma either treated or untreated were examined for expression of the isoenzyme of glutathione-S-transferase (GST), P-glycoprotein and p-21 on plasmacells, by immunocytochemical detection.
A high percentage of the evaluated samples was found to be positive (63% for P-170 and 66% for GST expression) without any correlation with clinical or prognostic parameters.
The percentage of positive plasmacells was not different in treated or untreated patients or in patients treated with different antiblastic agents.
GST expression was significantly related to the previous administration of cycles of chemotherapy ($p=<0.05$). A significant correlation between GST and P-170 reactivity was found ($p=<0.02$).
Expression of p-21 did not result associated to these mechanisms of drug resistance.

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BCL-2 ONCOPROTEIN EXPRESSION IN ACUTE MYELOID LEUKAEMIA

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A variety of cancer chemotherapeutic drugs and other agents are able to initiate pathways leading to apoptosis, or programmed cell death (PCD), in cancer cells. Previous studies have shown that Bcl-2, a mitochondrial oncoprotein, contributes to the promotion of cell survival by interfering in PCD. High levels of Bcl-2 oncoprotein may protect cancer cells from the acute cytotoxicity of multiple drugs commonly used in the treatment of acute myeloid leukemia (AML). The expression of Bcl-2 oncoprotein and its correlation with *in vitro* survival of leukemic cells maintained in liquid culture in the absence of growth factors and with *in vitro* adriamycin cytotoxicity were studied under different experimental conditions in peripheral blasts from 64 patients (28 males and 36 females) affected by AML at onset. More than 90% of each sample were leukemic blasts. A monoclonal antibody, anti Bcl-2 clone 124, was used to detect Bcl-2 expression by flow cytometric and immunocytochemical techniques. In 68% of the cases, Bcl-2 was found in more than 20% of blasts. In percentage terms, the highest levels of Bcl-2 expression were recorded in FAB subtypes M0, M3 and M4, while no expression was seen in our two M6 cases. An intermediate percentage of Bcl-2 expression was observed in the FAB subclasses M1, M2 and M4 eosinophil variant. Liquid culture assay significantly correlates with Bcl-2 oncoprotein, except in some cases of M3 subtypes. The cytotoxic effect of adriamycin was tested by blast culture 24h after drug exposure. Apoptosis was detected by morphological and flow cytometric methods, and compared with a control culture without the drug. The samples with high levels of Bcl-2 oncoprotein demonstrated a lower percentage of apoptotic cells following culture with adriamycin. The addition of verapamil, which blocks the P-170 glycoprotein responsible for multidrug resistance (MDR), did not increase the percentage of apoptotic cells in the presence of adriamycin. We suggest that Bcl-2 oncoprotein reduces the cytotoxic effects of several chemotherapic drugs by inducing prolonged cell survival. Blocking P-170 glycoprotein does not seem to enhance apoptosis induced by adriamycin. Bcl-2 and MDR thus probably induce two different drug resistance mechanism.

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TAXOL AND CROSS-RESISTANCE WITH ANTHRACYCLINES: AN IN VITRO STUDY

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Taxol is the prototype of a new class of antitumor agents that has significant activity in several human malignancies.
The high activity demonstrated in the treatment of breast carcinoma resistant to anthracyclines suggests a lack of cross-resistance with multidrug resistance (MDR) related-drugs in clinic (1). However, some authors (2) reported "*in vitro*" cross-resistance between taxol and other agents for which resistance is thought to be at least partly due to P-glycoprotein-mediated pleiotropic drug resistance. The aim of our research is to evaluate taxol activity, presence of cross-resistance and possible modulation in sensitive and multi-drug resistant cell lines *in vitro*. We utilized two erythroleukemia cell lines characterized for MDR phenotype (K562/DXR and the murine Friend leukemia FLC/DAU) and their parental counterpart. Taxol IC50 evaluated on K562 cells was 6.8 nM, while it was 2150 nM in K562/DXR (resistance index: 316). In FLC line taxol IC50 was 3.9 nM, while in the resistant cell line IC50 was 765 nM (resistance index: 196). In order to reverse *in vitro* taxol resistance, we employed verapamil, a classic inhibitor of P-glycoprotein activity and lacidipine (3), a dihydropiridine calcium antagonist showing high therapeutic index in clinic. Both modulators, at non cytotoxic concentrations, are able to partially restore taxol sensitivity in the resistant cells. MDR reversal is obtained by lacidipine at lower concentration than verapamil. These results confirm the existence of cross-resistance between taxol and anthracyclines *in vitro* and indicate the possibility to circumvent MDR with molecules able to interact with P-glycoprotein and characterized by low toxicity such as lacidipine.

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INCIDENCE AND CLINICAL RELEVANCE OF NEUTRALIZING ANTI-INTERFERON ANTIBODIES IN PATIENTS WITH PH+ CHRONIC MYELOID LEUKEMIA (Ph+ CML) TREATED WITH INTERFERON α2A (IFNα2A)

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By using a neutralization bioassay, the frequency of neutralizing anti-interferon α2a antibodies (anti-IFNα2a Abs) was evaluated in 67 Ph+ CML patients (pts) before, during and after discontinuation of IFNα2a therapy (average dose from 6 to 9 MU/day). The results are here reported:

	pts-studied	positive	negative
Prior to treatment	7	/	7
During IFNα2a treatment	41	7 (17%)	34
-pts-(H/K)-unresponsive		6/7	6/34
After discontinuation of IFNα2a (mo. mean 8.5)	19	5 (26%)	14
Total cases studied	67	12 (18%)	55

Out of 41 pts studied during IFNα2a treatment, 7 pts (17%) developed anti-IFNα2a Abs (title ranging from 1:10 to 1:20480) and nearly all of them (6/7) were hematologically and karyotypically unresponsive to therapy. Out of 19 pts studied after the discontinuation of IFNα2a treatment (mean=8.5 months), 5 pts (26%) were anti-IFNα2a Abs positive. Out of them 3 pts were previously positive for anti-IFNα Abs and discontinued IFNα2a therapy because of the lack of hematologic response; the other 2 pts stopped IFNα2a because of toxicity (grade III-IV).
Five pts who where anti-IFNα Abs positive and unresponsive to IFNα2a were crossed to treatment with lymphoblastoid IFNα (9MU/day). A complete hematologic response and a significant reduction of anti-IFNα2A Abs' title were observed in 3/5 pts who were evaluable at the 3rd, 6th, 9th and 12th month. Our results show that a non negligible percentage of Ph+ CML patients receiving a chronic treatment with IFNα2a develop neutralizing anti-IFNα2a Abs which are associated to a loss of IFNα2a efficacy. A change in therapy by using a non cross-reactive type of IFN-α should be considered in these patients.

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GENE THERAPY FOR DRUG-INDUCED MYELOTOXICITY: INDUCTION OF CYCLOPHOSPHAMIDE RESISTANCE BY ALDEHYDE DEHYDROGENASE-1 GENE TRANSFER.

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The dose-limiting toxicity of most anticancer drugs is represented by myelosuppression. At present, the strategies to circumvent myelotoxicity are based on hematopoietic growth factors infusion often in association with autologous bone marrow or peripheral blood progenitor/stem cells reinfusion. An alternative approach would be to make progenitor cells resistant to chemotherapeutic drugs by introducing specific drug-resistance genes.
It is well documented that resistance to the alkylating agent cyclophosphamide correlates with the expression of the cytosolic aldehyde dehydrogenase gene (Aldh-1). In hematopoietic cells, the Aldh-1 gene is down regulated during differentiation resulting in committed progenitor cells more sensitive to cyclophosphamide than their precursors. Based on these observations, we have designed a retroviral vector carrying the full length Aldh-1 cDNA, previously isolated from a liver cDNA expression library. The retroviral construct was introduced into the amphotropic packaging cell line PA317, and the resulting virus producer clones have been used to infect hematopoietic cell lines.
We have been able to achieve survival of Aldh-1-transduced murine (L1210) and human (K562) cells in the presence of concentrations (5-10 mM) of maphosphamide (a cyclophosphamide analog) comparable to those achieved *in vivo*. Maphosphamide-resistance could be reversed by treatment with disulfiram, an inhibitor of cytosolyc Aldh-1, indicating that it was specifically generated by Aldh-1 expression. These data demonstrate a direct relationship between Aldh-1 expression and cyclophosphamide-resistance and suggest that the Aldh-1 gene may be useful in generating hematopoietic progenitor/stem cells resistant to cyclophosphamide *in vivo*.

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EFFICIENT TRANSFER OF SELECTABLE AND MEMBRANE REPORTER GENES IN HEMATOPOIETIC PROGENITOR AND STEM CELLS PURIFIED FROM HUMAN PERIPHERAL BLOOD

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We have utilized highly purified hematopoietic progenitor and stem cells (HPCs, HSCs) from normal peripheral blood (PB) to develop methodology for: (i) efficient transfer into HPCs of a non-hematopoietic membrane reporter, i.e., the nerve growth factor receptor (NGFR) cDNA, (ii) effective gene transduction of putative HSCs, i.e., cells initiating Dexter-type long-term culture (LTC-ICs).
Purified HPCs induced into cycling by GFs [IL-3, IL-6, c-kit ligand (KL)] were transduced with the N2 retroviral vector containing the neomycin resistance (neo^r) gene: > 80% of transduced HPCs were resistant to toxic G418 level. Thereafter, the HPCs were effectively transduced with the LNSN retroviral vector containing a NGFR cDNA: the NGFR was detected on 18% of the transduced HPCs. These experiments provide a new tool to (i) monitor expression of a transduced membrane reporter on hematopoietic cells, particularly at the level of HPCs/HSCs and (ii) characterize the transduced cells by double-, triple-labeling membrane antigen analysis.
Purified HPCs/HSCs grown in Dexter-type LTC were transduced at 1 wk by exposure to supernatant N2 retroviral particles in the absence of exogenous hematopoietic GFs. The procedure, devoid of toxic effects, allowed an efficient neor transduction into LTC-ICs. Thus, we consistently detected neor mRNA in the clonal progeny of HPCs produced in LTC at 5-8 wk in both the non-adherent and adherent fractions: this timing of expression coincides with that of HPC production by LTC-ICs, thereby indicating the effective transduction of the LTC-ICs. These experiments represent a first step towards development of pre-clinical models for gene transfer into human PB HSCs by complex retroviral vectors.

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RETROVIRUS-MEDIATED TRANSFER OF THE MULTIDRUG RESISTANCE GENE INTO HUMAN HEMATOPOIETIC PROGENITOR CELLS

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Cord blood (CB) or bone marrow (BM) derived low density or purified CD34+ cells were evaluated as a target for human multidrug resistance (MDR1) gene transfer. Cells were cocultivated for 48-72 hours with 3,500 rads irradiated A12M1 retroviral producer cells. Since some degree of MDR1 gene expression is known to occur in hematopoietic progenitor cells and in peripheral blood cells, efficiency of MDR1 gene transfer was assessed as follows: 1) drug selection and culture in presence of 50 ng/mL doxorubicin, 10 ng/mL colchicine or 0.85 ug/mL taxol. In uninfected controls, 1-2% of CFU-GM and CFU-GEMM were found to be viable after drug selection, while 14-31% of seeded CFU-GM and CFU-GEMM were found to be drug-resistant and viable after 2 weeks of culture of transduced cells. Efficiency of MDR1 transfer was significantly enhanced by prestimulation with SCF and IL-3, and found to be superior in CB-derived versus BM-derived progenitors ($p<0.01$); 2) analysis of MDR1 gene expression by RT-PCR. MDR1 expression was undetectable in irradiated A12M1 cells after two week culture, undetectable or very low in fresh CB and BM cells and in cultures of uninfected controls. Faint MDR1 mRNA bands were observed after cocultivation, whereas after drug selection MDR1 mRNA levels in transduced cells was as high as in A12M1 retroviral producer cells evaluated as positive controls; 3) flow cytometric analysis of the expression of CD34 and P-glycoprotein, the product of the MDR1 gene. After MDR1 transduction and two weeks of drug selection, membrane expression of P-glycoprotein was found on 17-25% of viable CD34+ cells versus 3% of uninfected controls; 4) cytochemical localization by APAAP staining of P-glycoprotein. No specific localization was found in uninfected controls, while infected and cultured CB-cells expressed P-glycoprotein on plasma and nuclei membrane. The staining was exclusively seen in medium to small sized cells, but never observed in more mature myeloid cells and in macrophages.
In conclusion, MDR1 gene transfer into CB- and BM-derived progenitor cells seems feasible and a promising approach to generate a drug-resistant hematopoiesis.

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ADENO-ASSOCIATED VIRUS 2-MEDIATED GENE TRANSFER INTO HEMATOPOIETIC PROGENITOR CELLS IN HUMAN UMBILICAL CORD BLOOD

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Adeno-associated virus 2 (AAV) have been recently reported as suitable vectors for gene transfer in murine hematopoietic progenitor cells. We report here the construction of a hybrid recombinant vector between the AAV-2 and the parvovirus B19 which shows remarkable features in stably transducing specific genomic sequences into human hemopoietic progenitor cells.
AAV vectors were constructed containing the gene for resistance to neomycin, under the control of either the herpesvirus thymidine kinase (TK) gene promoter or the human parvovirus B19 p6 promoter, as well as containing an upstream erythroid cell-specific enhancer (HS2) from the locus control region of the human β-globin gene cluster. These vectors were used to infect either low density monuclear cells or a highly enriched population of CD34+ cord blood cells.
Whereas only 12% of CFU-GM colonies survived to 600 μg/mL G418, the percent survival of the CFU-GM colonies cloned from the AAV-Neo virions was 25% with TK-Neo promoter, 38% with HS2-TK-Neo, 39% with B19-Neo and 27% with HS2-B19-Neo. A similar pattern was observed for CFU-GEMM colonies.
Stable integration of the transduced Neo-R gene was documented by Southern blot analysis performed on individual CFU-GM, BFU-E and CFU-GEMM colonies.
Interestingly, pre-exposition of cord blood cells to IL-3+ GM-CSF+ SCF for 48 hours before infection with the AAV vectors did not affect the high efficiency gene transduction by this system ($p>0.05$).
This study suggests that AAV-mediated vectors may prove to be a useful alternative to the more commonly used retroviral vectors for high efficiency gene transfer into slow or non-cycling hematopoietic progenitor cells, without the need for growth factor pre-exposition, which could potentially lead to differentiation of these cells before transplantation.

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HUMAN LEUKEMIC CELLS TRANSDUCED WITH THE TNFα GENE CAN RESTORE THEIR APOPTOTIC CELL DEATH PROGRAM

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Apoptosis is a physiological program of cell death, that is usually lost during neoplastic progression. Since evidence has been presented that tumor necrosis factor α (TNFα) can induce tumor cell death by apoptosis, using a retroviral vector we have transduced the TNFα gene into human tumor cells to investigate whether through this approach the indefinite neoplastic proliferation could be blocked and the lost physiological program of cell death restored.
Our studies show that: a) a human leukemic T-cell line (ST4) transduced with the human TNFα gene can undergo apoptosis, and internucleosomial cleavage of DNA is detectable either by May Grünwald-Giemsa staining or by Propidium Iodide staining or by gel electrophoresis; b) the phenomenon can be restrained by incubation with an anti-TNFα monoclonal antibody, proving that the process is mediated by TNFα; c) the cytokine concentration released by the engineered cells, sufficient to induce apoptosis, is much lower (> 100 times) than that required with the administration of exogenous recombinant TNFα; d) the phenomenon is associated with a down-regulation in the expression of a gene involved in preventing apoptosis (bcl-2), while the expression of genes usually involved in promoting apoptosis (bax and p53) persists; e) TNFα-transduced ST4 cells are capable of triggering an apoptotic cell death program also in ST4 parental cells co-incubated in a mixed culture, suggesting possible clinical applications to control the expansion of a neoplastic clone *in vivo*. These findings point to a regulatory role of TNFα in the proliferation of human tumor cells and to a new therapeutic strategy worthy of investigation.

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FUNCTIONAL RECONSTITUTION OF THE NADPH OXIDASE ACTIVITY IN B-LYMPHOBLASTS FROM PATIENTS WITH X-LINKED CGD BY RETROVIRUS-MEDIATED GENE TRANSFER

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Chronic granulomatous disease (CGD) is an inherited hematological disorder characterized by the inability of phagocytic leukocytes to generate microbicidal toxic oxigen metabolites due to a failure of a specialized NADPH oxidase.
Most of the cases of CGD result from mutations in the X-linked gene encoding for gp91-*phox*, the β subunit of the cytochrome b of the phagocyte oxidase complex. In this study we have addressed the feasibility of a gene therapy protocol for X-CGD by retroviral-mediated gene transfer.
Three patients with X-CGD were enrolled in the study; single point mutations, not yet described, were found to be responsible for the defect in all the three patients, as determined by cloning and sequencing the gp91-*phox* cDNA.
We have used EBV-transformed B cell lines established from the patients as a model to test the feasibility of genetic reconstitution. The Moloney-based retroviral vector pBabeHygro was used to transduce the gp91-*phox* cDNA coding region into the lymphoblastoid cell lines of two patients; several clones were selected and isolated, and the restoration of the oxidase enzymatic funtion was tested with a highly sensitive chemiluminescent assay. All the pBabe/gp-91*phox* clones analyzed showed a substantial oxidase activity.
Evidence was provided for a great clonal variability in the expression of the gp91-*phox* gene, with values ranging from 2% to 30% of the activity of a reference normal B-cell line.
This study demonstrates that the X-CGD defect can be successfully corrected by a retrovirus-mediated gene transfer approach and encourages the utilization of the pBabeHygro/gp91-*phox* vector for the infection of the hematopoietic stem cells of the patients.

063

CLINICAL SIGNIFICANCE OF BCL-2 EXPRESSION IN ACUTE MYELOID LEUKEMIA

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The BCL-2 proto-oncogene encodes a mitochondrial protein that blocks programmed cell death. Using a monoclonal antibody (MoAb) against the bcl-2 molecule, staining of normal bone marrow myeloblasts, promyelocytes, and myelocytes was demonstrated (*Delia et al.,1992*). The bcl-2 protein levels among normal and malignant myeloid cells seem to be inversely related to the maturation stages (*Delia, 1992*). Campos et al. (1993) showed that the binding of the anti-bcl-2 MoAb to leukemic cells was heterogeneous in terms of percentage and fluorescence intensity, which makes it difficult to define two groups, one positive and one negative. Our study included 27 consecutive patients with acute myeloid leukemia (AML), 19 *de novo* and 8 in first relapse; median age was 56 yrs, 14 were males and 13 females. All patients were treated by intensive chemotherapy including an anthracycline, etoposide and high doses of cytosine-arabinoside. Bcl-2 expression was analyzed using the FITC-conjugated MoAb bcl-2/124, IgG1 isotype, by flow-cytometry technique. Bone marrow and/or peripheral blast cells were permeabilized in 3.5% paraformaldehyde/PBS for 10 min at room temperature and, after two washings in PBS, in 50% cold acetone/PBS at 4°C. Cells were then incubated for 30 min at 4°C with 10 μL of bcl-2 FITC MoAb (Dako, Glostrup, Denmark). Negative controls were performed by incubating cells with non specific isotype-matched MoAb. We arbitrarily chose to consider AML with 40% or more stained cells as positive because it was necessary to distinguish two categories for statistical analysis; however, the use of flow cytometry (Epics Profile, Coulter, USA) allowed us to quantify easily either the percentages or the mean fluorescence intensities of positive cells. Twenty-four of 27 samples (88.5%) contained 40% or more stained cells. The mean fluorescence intensity of staining of bcl-2+ cells was significantly higher in FAB M0 and M1 cases ($P = 0.035$) and in AML expressing the CD34 phenotype ($P = 0.007$). Conversely, a CD14 positivity was correlated with a lower mean fluorescence intensity of staining ($P= 0.026$). There was no correlation with CD15, CD13, TdT, CD7 and CD56. The treatment outcome was not significant different among two positive groups for bcl-2, one with weak expression and one with strong intensity of staining. In conclusion, we show that bcl-2 is expressed in AML, and the mean fluorescence intensity more than the percentage of positive cells correlates with biologic findings such as immature FAB subtype and CD34 expression. The short follow-up of our patients doesn't allow us to evaluate the prognostic implications of bcl-2 phenotype.

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MESF CALCULATION OF PECAM, CR1, CD15, CD14 ANTIGEN EXPRESSION IN NORMAL AND MYELODYSPLASTIC SUBJECTS BY FLOW CYTOMETRY

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Myelodysplastic syndromes (MDS) are a heterogeneous group of disorders of clonal origin characterized by both morphological abnormalities and functional deficiencies. Great importance is given to the functional impairments of the physiological activities which involve the role of strategical cell to cell adhesion molecules. In this perspective flow cytometry may give an important contribution by means of the MESF (mean equivalent standard of fluorescence) calculation. The importance of MESF analysis relies on the possibility of defining the exact number of antigen molecules revealed by flow cytometry instruments and in the comparability of the obtained results not only within one laboratory but also between various laboratories using different instruments. This is obtained using a mixture of differently fluorescinated microbeads which are used both as standard of fluorescence and calibrating system. Ten non-smoker healthy subjects and ten patients affected by myelodysplastic syndromes were evaluated in single fluorescence indirect technique with a FACScan (Becton Dickinson) using 6 different monoclonal antibodies and the correspondent calibrating microbead kit, all provided within the Fifth Workshop on Human Leukocyte Antigens. M12 (CD31), M23 and M27 (CD15), MR4 (CD14), and M18 (CD35) were evaluated on the gated monocytic and granulocytic subpopulations. Linear regression was used to determine from the calibrating curve, the MESF conversion and subsequently the exact number of surface molecules by means of the specific monoclonal Protein/fluorescence ratio. Normal ranges of expression were determined by considering the normal subject pattern of reactivity. Monocytes and granulocytes derived from patients belonging to RAEB, RAEB-t and CMML subgroups were found to express a lower number of surface antigen molecules if compared with the normal population ($p<0.05$). In particular M12 was found to be nearly absent on MDS monocytes. MDS had a heterogeneous pattern of expression: a normal pattern of positivity was demonstrated in Refractory Anaemia granulocytes and monocytes thus confirming the erithroid mono-lineage involvement. In one case the diagnosis of myelodysplasia was confirmed 6 months after the cytofluorimetric analysis. We conclude that the cytofluorimetric analysis by means of the MESF calculation with the subsequent evaluation of the exact number of surface antigen molecules may be a useful tool in the study of the biology of MDS.

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POST REMISSION CHEMOTHERAPY WITH ABMT IN ACUTE MYELOGENOUS LEUKEMIA (AML)

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Aim. To evaluate the toxicity and long term results of ABMT performed in 1st remission AML.
Materials and methods. From April 1987 51 patients with AML were treated in our Institute with high dose chemotherapy and autologous bone marrow transplantation (ABMT).
Patient characteristics. 27 males, 24 females, mean age 36 yrs (range 17-59), median interval between CR and ABMT 8 months (range 4-20). Conditioning: busulphan (BU) 16 mg/kg in 4 days and cyclophosphamide (CY) 200 mg/kg in 4 days in 12 pts, BU 16 mg/kg in 4 days plus CY 120 mg/kg in 2 days in 39 pts. The mean dose of mononuclear cells reinfused was 1.43×10^8/kg (range 0.5-2.4). Median day to 500 PMN/mm^3 was + 26 (range 12-250), median day to 50,000 platelets/mm^3 was +102 (range 18-610). Mild hemorragic cystitis occurred in 2 patients; moderate to severe mucositis occurred in 14 patients (21%). No treatment-related deaths occurred.
Long-term results. Thirty-nine patients had at least one year follow-up. Six pts relapsed and dead 29, 23, 23, 10, 17 and 9 months after ABMT; one pt relapsed at 4 months and underwent 2nd CR after low dose ARA-C; she is now in CR after 80 months from ABMT. One pt relapsed at 7 months and achieved a 2nd CR after conventional chemotherapy, he is now in 3rd CR after a second relapse at 32 months from ABMT. One pt relapsed 11 months after ABMT and underwent a second CR after ATRA therapy. Thirty pts (77%) are alive in first CR with a mean follow up of 29 months (range 12-86). Seven years leukemia-free survival (LFS) curve is projected at 71%.
We have calculated the relapse curve in our population of 63 matched adults with AML treated with chemotherapy who had at least 8 months of CR: the shape of the curve is different from that observed after ABMT, with a high relapse rate for the first 36 months.
These results suggest that even if ABMT is performed late after CR it still gives a survival advantage compared to chemotherapy.

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INFLUENCE OF SOME KNOWN GROWTH (G- AND GM-CSF AND IL-3) AND DIFFERENTIATING FACTORS (TRANS-RETINOIC ACID AND THIOPROLINE) ON CFU FROM THE PERIPHERAL BLOOD OF NORMAL AND LEUKEMIC SUBJECTS (CML AND CMmL)

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The authors have studied the behavior *in vitro* of the peripheral CFU of some patients with CML or CMmL in basal conditions and in presence of G-and GM-CSF and IL-3 separately or in association with trans-retinoic acid (R) or thioproline (T). The colony growth, the cell proliferation, the cell morphology with the traditional staining of the smeared elements, and the following cell markers Cd11, Cd13, Cd14, Cd15, Cd34 were controlled.
In basal conditions, without stimulation, the peripheral CFU of CML shows only a modest increase of cell number in culture, while the appearance of fibroblasts is very uncommon. R enhances the colony growth particularly in presence of GM-CSF and IL-3, while T seems responsible for an evident increase of fibroblasts. The morphologic examination of cultured cells shows a longer persistence of granulated blasts in presence of GM-CSF and IL-3, partially counteracted by R and T. The control of cell marker partially confirm these observations and signalled a mean increase of the adhesion molecules with the growth factors, R and T.
The cultures of peripheral CFU of CMmL presents in basal conditions (without any stimulation) the prevalent precocious formation of fibroblasts, while the growth factors employed increase the colony appearance of mean volume and fibroblasts, modestly counteracted by R, but enhanced by T. The cell markers varied similarly to CML cultured cells.
We conclude that growth factors does not influence negatively the behaviour *in vitro* of peripheral CFU from CML and CMnL, particularly in association with R or T.

067

DIFFERENTIATING AGENTS AND LOW-DOSE 6-THIOGUANINE FOR POOR PROGNOSIS MYELODYSPLASTIC SYNDROMES AND ACUTE MYELOID LEUKEMIA

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Myelodysplastic (MDS) patients presenting with bone marrow (BM) blast excess have a short survival (median 6-18 months), slightly modified by the currently available therapies. A similar, if not poorer, outcome characterizes acute myeloid leukemia (AML) patients older than 65 years or with refractory disease. On the basis of previously reported positive interactions among differentiating agents and 6-thioguanine (6TG) or cytosine arabinoside, we treated 26 MDS patients with poor prognosis (17 RAEB, 6 RAEB-T, 3 CMMoL) with a combination of cis-retinoic acid (Roaccutan, Roche) 20-40 mg/day, 1;25_$(OH)_2$ vitamin D3 (Rocaltrol, Roche) 1-1.5 ug/day and 6TG (Thioguanine, Wellcome) 20-60 mg/day for 3 weeks every 5 weeks. The therapy was administered for at least two courses, then continued in case of response or stable disease. No major toxicities occurred. One patient obtained a complete hematological response (CR) (normal blood counts and BM blasts < 5%), 11 a partial response (PR) (defined as at least 50% BM blast reduction and/or 50% improvement of peripheral blood cytopenia) and 5 a minor response (MR) (defined as 25-50% reduction in BM blasts and 25%-50% improvement in blood cytopenia), for an overall response rate of 65%. No significant differences in responses were observed between the group with BM blasts of 5-10% and the group with BM blasts of 11-30%. The median response duration was 8 months (2-29+). After a median follow-up of 24 months, the overall median survival is 16 months (in particular, 25 for responders, 9 for non-responders). The same treatment was used for 16 AML patients. Eight were not eligible for conventional chemotherapy because of old age and/or concurrent diseases, whereas eight were at second or subsequent relapse. One patient (previously untreated) obtained a CR, 6 a PR and 3 a MR (total response rate 56%). The median survival was 7.5 months (11+ for responders, 3 for non-responders) with a range of 1-15+. No treatment related death occurred.
In conclusion, this protocol seems capable of inducing hematological improvements in 50-60% of patients with poor prognosis myeloid neoplasia. A larger study is warranted to assess its actual impact on survival.

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RAPID DETECTION OF CLONAL B CELL PROLIFERATION IN BLOOD AND TISSUE SAMPLES BY POLYMERASE CHAIN REACTION

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A clue in the laboratory diagnosis of lymphoproliferative diseases is provided by molecular biology techniques capable to detect clonal rearrangement of immunoglobulin and T-cell receptor genes. Hybridization with specific probes in Southern blot analysis is the most widely used method, but it has some disadvantages: it requires large amount of fresh or frozen DNA sample and a few days to obtain the final result. Furthermore it employs radioactively labelled probes which adds unnecessary complexity to the routine activity of a diagnostic laboratory. Polymerase chain reaction (PCR) has been recently proposed as an alternative approach to the analysis of immunoglobulin genes rearrangement. This method relies on the use of consensus primers, which hybridize with conserved sequences present in most of the variable (V), diversity (D) and joining (J) regions of the genome. Since the rearrangement process inserts a random sequence of bases, named "N" regions, between V, D, and J segments, the amplification of these sequences by PCR from a normal polyclonal cell populations generates a large number of different size DNA segments; on the contrary, a clonal populations generates a large number of identical DNA segments, reflecting the single gene rearrangement present in all the cells of the clone. On this basis, DNA is detectable as a single band on agarose gel in the case of clonal amplification, while no band or a smear will be visible if dealing with a polyclonal cell population. We analyzed 58 cases of B-cell lymphoproliferative disease using 2 sets of oligonucleotides. Every sample was preliminarily examined with a single round PCR; if clonal rearrangement was not detected, DNA sample was subjected to a seminested PCR employing the other set of primers. Using this approach, monoclonal rearrangement was detected in 80% of lymphoproliferative disease examined. The most striking result was that many samples were derived from fine needle aspiration (lymph nodes, peri-ocular tissues), low yield biopsies (gastric and cutaneous). In these very cases, this approach provides a very useful tool – and probably the only one – for the rapid clonal analysis of lymphoid proliferations when tissue sample are provided in very low amount.

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IN VITRO AMPLIFICATION OF CD34+ CD45RA^{LO} HEMATOPOIETIC PROGENITOR CELLS

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CD34+ CD45RA^{lo} cells purified from adult bone marrow, cord blood and fetal liver have been shown to be functionally different with respect to their proliferative potential. No information is so far available on the proliferative potential of circulating CD34+ CD45RA^{lo} cells. It, therefore, was the aim of the present study to purify and characterize CD34+ CD45RA^{lo} cells from peripheral blood of normal donors or patients undergoing mobilization of circulating progenitor cells for therapeutic procedures.
Peripheral blood cells were fractionated by density-gradient centrifugation (Ficoll-Hypaque, d=1,077 g/mL) and depleted of soybean agglutinin (SBA) positive cells, through an immunoadsorption technique using flasks (AIS, MicroCELLector™) coated with SBA. The SBA-neg cells were labeled with an anti-CD34 (HPCA-2_{PE}, Becton-Dickinson) and an anti-$CD45RA_{FITC}$ (B-D) monoclonal antibodies (moabs). CD34+ CD45RA^{lo} cells were obtained by fluorescent-activated cell sorting (FACSort, B-D).
Flow-sorted cells were >95% CD34+ and CD45RA^{lo}. Triple labeling with PerCP-conjugated moabs, revealed that this cell fraction was negative for the lineage-specific markers CD19, CD7, CD33. Culture of CD34+ CD45RA^{lo} cells in semi-solid media supplemented with stem cell factor (SCF), interleukin-3 (IL-3), granulocyte-macrophage-CSF (GM-CSF), granulocyte-CSF (G-CSF) and erythropoietin (Epo) showed a plating efficiency ranging from 15 to 30%. CD34+ CD45RA^{lo} cells were capable to initiate and sustain hematopoiesis in long-term culture. Suspension culture of CD34+ CD45RA^{lo} cells over a period of 7-21 days in the presence of SCF, IL-3, IL-6, Epo resulted in a large increase (3 to 4 logs) in the total number of cells and a 8-25-fold increase in multipotential (CFU-Mix), erythroid (BFU-E) and granulocyte-macrophage (CFU-GM) progenitors.
In conclusion, our data demonstrate that circulating CD34+ CD45RA^{lo} cells obtained by flow-sorting: (a) have a high progenitor cell content; (b) are capable of initiating and sustaining hematopoiesis in long-term culture; (c) are susceptible of *in vitro* amplification. The potential clinical implications of this procedure require further investigations.

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POSITIVE SELECTION AND REINFUSION OF AUTOLOGOUS HEMATOPOIETIC CD34+ CELLS AFTER MYELOABLATIVE THERAPY

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In this study, the Ceprate SC Stem Cell Concentrator (Cell Pro), an automated version of the original avidin-biotin cell separator system, was used to enrich hematopoietic CD34+ cells from the bone marrow (BM)(=4) or peripheral blood (PB)(=4) of lymphoma or myeloma patients (pts), respectively. Circulating CD34+ cells were collected after mobilization with cyclophosphamide (7 g/m^2) or VP-16 (2 g/m^2) followed by G-CSF (5 ug/kg/day). In all cases, additional BM cells or apheresis products were cryopreserved as unmanipulated back up. The results of CD34+ cells positive selection are presented in the Table.

Pz	Diagn.	Source	CD34+ cells×10^6/Kg				CD34+ purity	CFU-GM×10^4/Kg				Take	
			Pre	Post	Reinf	Rec%	%	Pre	Post	Reinf	Rec%	>500 PMN	>20.000 plt
PL	LH	BM	0.7	0.39	0.54	77	84	1.7	1.4	0.94	55	14	34
TS	LH	BM	1.9	0.86	1.14	60	89	1.37	1.2	1.14	83	14	20
AA	LNH	BM	1.9	0.92	0.96	50	87	1.17	0.6	0.5	43	11	9
MD	LNH	BM	1.1	0.32	/	29	64	1.2	0.53	/	44	/	/
BR	MM	PBC	4.6	3.23	2.98	65	83	10.3	2	0.67	7	11	17
CM	MM	PBC	4.8	1.77	2.5	52	80	7.3	9.1	7.34	100	10	32
MC	MM	PBC	12	9.37	/	78	91	37.8	18.2	/	48	/	/
NL	MM	PBC	6.1	4.38	/	72	87	38.5	23.8	/	62	/	/

The recovery of hematopoietic precursors of the patients reinfused with CD34+ cells refers to thawed samples.

The purity of CD34+ cell population was in all cases over 60% with a 78 fold increase compared to the pre-treatment samples (95-fold increase in the thawed samples). The mean viability of selected cells by trypan bleu dye exclusion and propidium iodide was 79% (range 54-100%). The median overall recovery of CD34+ and CFU-GM was 63% (range 29-78) and 52% (range 7-100) respectively. The engraftment data after myeloablative therapy did not differ significantly from those of patients reinfused with untreated stem cells.
In conclusion, the preliminary results of this phase I study demonstrate the capacity of Ceprate SC to enrich hematopoietic progenitor cells which are then able of reconstituting a stable hematopoiesis after myeloablative radio-chemotherapy.

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HIGH-DOSE CYTOXAN (HD-CY)+G-CSF PRIMED PERIPHERAL PROGENITOR CELLS (PPC) AND AUTOGRAFT FOR NON-HODGKIN'S LYMPHOMA (NHL) WITH PERSISTENT BONE MARROW INVOLVEMENT

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Twenty seven patients with intermediate or high-grade NHL, pretreated with a median of two combination chemotherapy lines (range 1-4), but not in CR because a persistent BM involvement (5 to 50%; median 15%), underwent HD-CY (7 gr/sm,single dose) and G-CSF infusion (5 ug/kg/day, continuous infusion) in order to reduce tumor burden and to collect PPC. The median age of pts. was 43 yrs. (range 19-54); 17 pts. were males and 10 females. Twelve pts. presented also nodal disease. The collection began with a median number of about 1.000/mcl WBC. Median number of apheresis was 6 (range 3-12); median collected cells were 6.9×10^8/kg (range 2.7-13); median of CFU-GM was 7.7×10^4/kg (range 0-71.3) and of CD34+/CD33– was 4.4×10^6/kg (range 0-28).
In 25 patients, phenotypic analysis of collected cells showed values to be within the normal range. In one patient, a clonal B lineage population developed (CD10+=24-43%, CD19+=36-54%). In another patient, peripheral and BM progression occured during collection. Two patients did not undergo PPC rescue because a low number of CFU-GM. After a conditioning regimen (melphalan 120 mg/sm + TBI, or BEAM), 21 pts. received PPC rescue, and 20 are evaluable. Seventeen out of 20 pts. (85%) obtained BM and nodal complete remission, while three were non responder. Overall short-time procedure was well tolerated.
However, 5/20 pts.(25%) died in CR (1 of lung fibrosis at 4 mos., 2 of broncopneumonia at 2 and 5 months, and 2 of BM aplasia at 3 and 7 months after rescue). Four patients relapsed at 6,7,8 and 9 months. Up to now 8/20 pts.(40%) are in CR 6 to 22 mos.(median 10) after PPC rescue.

072

POSITIVE SELECTION OF MOBILIZED CD34+ CELLS PROVIDES INDIRECT PURGING OF CIRCULATING TUMOR CELLS IN MULTIPLE MYELOMA (MM) PATIENTS

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One advantage of the use of peripheral blood stem cells (PBSC) over autologous bone marrow (BM) would be a reduced risk of tumor cell contamination. However, the level of neoplastic cells in the PB of MM patients is poorly investigated. We evaluated PB samples from 14 MM patients following the administration of high dose cyclophosphamide (7 g/m^2 or 4 g/m^2) and G-CSF for the detection of neoplastic plasmacells and their precursors. Cells containing clg were counted by immunofluorescence after incubation with appropriate goat antisera directed against light and/or heavy chain Ig. Moreover, to determine the presence of immature B-lineage elements, monoclonal antibodies against the CD19, CD34 antigens and p-170 glycoprotein (multi-drug resistence associated protein) were used in an immunocytochemical assay or immunofluorescence. Before initiation of PBSC mobilization, circulating tumor cells were detected in all MM patients in a percentage ranging from 0.5 to 1.8% of the mononuclear cell fraction. In these patients, a higher absolute number of PB neoplastic cells was detected after chemotherapy and G-CSF. Kinetic analysis showed a pattern of tumor cell mobilization similar to that of normal hematopoietic progenitors with a maximum peak falling within the optimal time period for the collection of PBSC. Apheresis products contained 0.29-0.9% of myeloma cells. In 4 patients the Ceprate SC Stem Cell Concentrator (Cell-Pro), an automated version of the original avidin-biotin cell separation system, was used to enrich circulating hematopoietic CD34+ cells. The purity of CD34+ cell population was always over 80% with a 78-fold increase compared to the pre-treatment samples. The median overall recovery of CD34+ cells and CFU-GM was 68.5% (range 52-78%) and 55.5% range (7-100%). Positive selection of CD34+ cells did also result in 2.5-3 Log of tumor cells depletion. The patients (= 3) reinfused with purified hematopoietic progenitor cells after myeloablative radio-chemotherapy showed a rapid reconstitution of BM function. In conclusion, our data suggest the concomitant mobilization of tumor cells and hematopoietic progenitors in the PB of MM patients. Furthermore, positive selection of CD34+ cells is able to remove myeloma cells from the apheresis products and provides a cell suspension capable of restoring a normal hemopoiesis after a myeloablative conditioning regimen.

073

MULTIPLE MYELOMA: APPROACHING HIGH DOSE CHEMOTHERAPY ON AN OUT-PATIENT BASIS, THE SCM PROTOCOL

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In multiple myeloma patients, high-dose chemotherapy induces high remission rate and improves survival. Such treatments are limited to young patients. I.V. L-PAM is the most effective drug, but its wide applicability is hampered by prolonged myelosuppression. To overcome these difficulties, a novel approach with sequencial cyclophosphamide, melphalan (SCM protocol) has been explored. The aim was to develop a safe, less costly, widely applicable protocol with limited hematological toxicity. Twelve refractory myeloma patients received CTX followed by G-CSF, leucaphereses, L-PAM and reinfusion of unprocessed non-cryopreserved circulating progenitor cell (CPC) support.

day							
0	+3	+9	+10	+11	+12	+14	+23
CTX	G-CSF		STEM CELL	L-PAM	STEM CELL	G-CSF	
2 g/m	10 μg/Kg		HARVEST	60 mg/m	INFUSION	5 μg/Kg	

The entire procedure was performed on an out-patient basis; the median age was 58 years (range 42-64). CPC were always harvested on day 10 with a median WBC count of 19.9×10^9/L (range 9-27), kept unprocessed at 4°C, and reinfused after 48 hours. Yields (mean±SEM) were: 5±1.8 10^8/kg for total nucleated cells; 3.7±0.6 10^6/kg for total CD34+ cells; 14.26±8.3 10^4/kg for CFU-GM. No hematological toxicity was observed after CTX. L-PAM determined: median duration of neutrophils <0.5 x 10^9/L 6 days (range 5-8), median duration of platelets <25×10^9/L 2 days (range 0-7). Platelet transfusion was required in 3/12 patients and red cell transfusion in 4/12. Pre-treatment hematological count was achieved after a median of 22 days (range 13-36) from L-PAM. As a result of the short myelosuppression, only 2/12 patients developed unexplained fever without sign of infection, one patient contracted interstitial pneumonia, two required hospitalization for major complications (fever and pneumonia). In conclusion, the SCM regimen allowed the delivery of an aggressive chemotherapy with excellent tolerability, suggesting further dose intensification and recruitment of older patients.The unprocessed non-cryopreserved CPC and the short myelosuppression allowed its administration on an out-patient basis with significant cost reduction.

074

DONOR LEUCOCYTE INFUSION AFTER CHEMOTHERAPY FOR RELAPSE OF ACUTE LEUKEMIA AFTER ALLOGENEIC BONE MARROW TRANSPLANTATION

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We decribe the effect of the infusion of donor peripheral blood leucocytes (PBL) after G-CSF administration, in 2 pts (age 20 and 29) affected by acute leukemia (ALL and AML-M2) relapsed 10 and 12 months after alloBMT and treated with aggressive chemotherapy (ACHT)(IDA-ARAC and MEC). Donor PBL were collected after G-CSF administration 16 ug/Kg/day sc for 6 days, starting leukaphereses (LKP) at day 3 of G-CSF treatment and reinfusing them at least 8 days after the end of ACTH,allowing the wash out of the anthracyclines. Two products of LKP were reinfused in the induction phase for both pts. A total of 5.6 and 6.3×10^6/Kg CD34+ cells, 2.7 and 3.0×10^4/kg CFU-GM and 4.7 and 4.4×10^8/Kg MNC were infused. No side effects were observed after G-CSF in both donors. Both pts achieved complete remission (CR) with a short period of neutropenia. PMN >0.5×10^9/L were reached after 23 and 18 days and PLTs >50×10^9/L after 25 and 48 days respectively. A second course of PBL was administered after achieving CR. Follow up is very short with both pts alive and in CR after 4 and 6 months respectively. One pt developed grade III aGVHD of liver after the second reinfusion of PBL and is currently requiring immunosuppressive therapy with cyclosporine A and Prednisone. Donor PBL infusions after ACTH for relapsed acute leukemia seems to be a very promising approach taking advantage of both the immediate effect of granulocyte transfusions in the early phase of neutropenia and of a late hemopoietic reconstitution substained by CD34+ cells. Moreover the immunomodulating activity of infused donor's lymphocytes may contribute to control of minimal residual disease.

075

ALLOGENEIC PERIPHERAL HEMOPOIETIC STEM CELLS (PHSCS) INFUSION REVERSES CHIMERA IN RELAPSED ALLO-BMT PATIENT.

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Relapse after allo-BMT is an adverse event associated with short survival and bad response to conventional chemotherapy. Different approaches have been proposed in the attempt to reverse chimera and prolong survival: a second BMT by using different conditioning regimen is generally associated with a high transplant related mortality particularly in pts. in whom relapse occurred within one year from the first BMT. Recently donor PBCs have also been employed in pts. transplanted for CGL with recurrence of their disease; in ANLL, their role in patients relapsed after transplantation is more controversial. Here we report on a case of ANLL in relapse after allo-BMT treated by conventional chemotherapy followed by donor`s PHSCs infusion. A 36-year-old man affected with ANLL in CR received allo-BMT from his HLA identical sister. Six months later fully hematologic relapse occurred. We decided to treat the patient by using a conventional chemotherapy schedule consisting of mitoxantrone 8 mg/m^2/day, etoposide 60 mg/m^2/day, cytarabine 1 g/m^2/day all given over 5 days. Two days after the end of chemotherapy the patient received from his donor PHSCs mobilized by subcutaneous injection of G-CSF at the dose of 5 ug/kg/day. The total number of collected mononuclear cells were 4.6×10^8/kg. The CD 34 positive cells were 2.3×10^6/kg, the CFU-GM cells were 10×10^4/Kg. The patient recovered the neutrophil absolute count of 0.5×10^9/L by day +12 and 1×10^9/L by day +13. Complete remission was documented on day +16, it was confirmed by cytogenetics which detected female metaphase only. FISH technique revealed 14% Y-spot positive cells. This percentage remained unchanged until day +36 when the patient developed a II grade acute GvHD. Immunosuppressive therapy with steroids and cyclosporine induced complete remission. At present (+150) the pt is alive and well the Y-positive cells investigated by FISH technique is only 3%. This report shows that the procedure reported here is well tolerated and it is able to induce remission by reversing chimera.

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TREATMENT OF ADVANCED LEUKEMIA WITH G-CSF MOBILIZED ALLOGENEIC PBSC

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Three patients (1 CML, 1 AML and 1 ALL) received G-CSF mobilized allogeneic PBSC from their HLA identical siblings. The first two patients were in relapse after allogeneic BMT, while the ALL patient was in 2nd CR after CNS relapse. To mobilize PBSC, the donors received rhG-CSF 16 μg/kg daily for 4 days (Fig.1). Leukaphereses were started on day 4 and performed over two consecutive days. rhG-CSF side effects were moderate with malaise, bone pain and myalgia; a moderate, decrease of platelet counts was observed during the apheretic collections. A median of 8.7×10^8/kg (range 5-10) nucleated cells, 80×10^4/kg (range 37-190) CFU-GM, 13.5×10^6/kg (range 6.8-23) CD34+cells and 2.6×10^8/Kg (range 2.5-3.4) CD3+ cells were collected. The two previously transplanted patients received the PBSC with no conditioning regimen: none showed cytopenia and moderate GVHD grade II was observed in one. The ALL patient was conditioned with BUCY and received CSA + MTX short course as GVHD prophilaxis. The median time to an absolute neutrophil count > 0.5×10^9/L and to platelet count > 50.0×10^9/L were respectivaly 12 and 13 days. Neither GVHD nor fever were observed. In conclusion rhG-CSF can be used in normal donors to easily mobilize the large amount of PBSC that are necessary for prompt engraftment. Clinical results in terms of both hematopoietic reconstitution and GVHD severity are encouraging.

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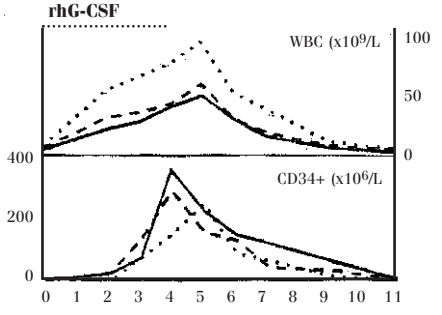

Figure 1. Variations of WBC and peripheral CD34+ cells after G-CSF priming in three normal donors.

077

ALLOGENEIC TRANSPLANT WITH PERIPHERAL BLOOD STEM CELLS IN PATIENT RELAPSED AFTER AUTOLOGOUS BONE MARROW GRAFT

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Peripheral blood stem cells (PBSC) are routinely used to restore autologous hemopoiesis following high-dose chemo-radiotherapy in hematologic malignancies and solid tumors. The capability of recombinant hemopoietic growth factors to mobilize PBSC has raised interest in using this technique for allogeneic transplant in man. We report a clinical case of successful second transplant with HLA identical related PBSC in one AML patient relapsed after autologous bone marrow graft. A 49-year-old male with M4 acute myeloid leukemia in first complete remission (CR) underwent autologous bone marrow transplantation (ABMT) 2 months after the end of conventional chemotherapy. The pre-transplant preparative regimen was busulfan 4 mg/kg/day for 4 days, etoposide 40 mg/kg for 1 day, cyclophosphamide 60 mg/kg/day for 2 days. The patient remained *disease-free* 14 months from ABMT then relapsed with 7% of marrow blasts evaluated by morphological method. Initially the patient was treated with Interleukin 2 (IL2) aimed at eradicating leukemic blasts by enhancing the cytotoxic activity of immunocompetent cells. The immunotherapeutical approach failed to achieve a second remission so the patient underwent allogeneic transplant with PBSC from his HLA identical sister. Hemopoietic cells were mobilized from donor by daily subcutaneous injection of G-CSF (10 ug/kg/day) for 5 days which resulted in an increasing number of leucocytes of 52,000/ul. On days 4-5 after the start of G-CSF, two leukapheresis yielded a total number of 3×10^{10} nucleated cells containig 4.75×10^8 CD34+ and 11.64×10^9 CD3+ cells. *In vitro* clonogenic assays of the nucleated cells revealed 3.23×10^7 CFU-GM, 2.44×10^7 BFU-E, 2.1×10^6 CFU-MIX. The conditioning regimen consisted of busulfan 4 mg/Kg/day (days - 7 to -5), thiotepa 10 mg/kg (day -4) and melphalan 90 mg/m^2 (day -2); as GVHD prophylaxis the patient received antithymocyte globulin (ATG) 5 mg/kg/day (days -5 to -2) and cyclosporine at standard dose (from day -1); 1.8×10^8/kg hemopoietic cells were reinfused on day 0 without T-cell depletion (4.2×10^6/kg CD 34+, 79.3×10^6/kg CD3+, 1.88×10^5/kg CFU-GM, 1.88×10^5/kg BFU-E, 1.1×10^4/kg CFU-MIX). The patient developed mild acute GVHD which was limited to the skin (grade I) at day 17+. Granulocyte recovery (> 500/uL) and platelet recovery (> 20.000/uL) occurred on day 17+ and 22+ respectively. Day 30+ bone marrow showed no evidence of malignant blasts and 100 % of hemopoietic donor-type cells were found by cytogenetic studies. The patient was discharged from the hospital on day 25+ and is now 52 days after PBSC with sustained engraftment. We have demonstrated that PBSC mobilized with G-CSF and harvested from healty donors may be used to achieve a rapid and complete engrafment. In this patient severe GVHD did not developed although non T-depleted cells were reinfused and no significant toxic effects were observed during post transplant clinical course. This report does not permit conclusions regarding the futur outcome of the patient in term of disease free survival but we suggest that a second transplant with allogeneic PBSC is a feasible treatment where a primary autologous graft is unsuccessful.

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A PHASE I STUDY OF HUMANIZED ANTI-TAC (HAT) IN PATIENTS WITH ACUTE GVHD REFRACTORY TO CYCLOSPORINE AND CORTICOSTEROID

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Patients undergoing allogeneic BMT frequently develop acute-GvHD, which represents the primary or contributing cause of early death in many cases. The mortality rate is particularly high for patients with advanced grade A-GvHD refractory to conventional treatment with cyclosporine (CSA) and steroids. Donor T-cells activated by minor HLA antigens on host tissues play a key role in the mechanisms of A-GvHD. Therefore, several murine anti-IL-2 receptor antibodies have been administered in clinical trials to treat A-GvHD with promising results. However, the therapeutic efficacy of rodent monoclonal antibodies is limited by the immune response of the recipient to the heterotopic protein. The humanized anti-TAC (HAT) is a genetically engineered human monoclonal antibody which binds to the p55 subunit of the IL-2 receptor. The immunogenicity of the HAT is expected to be less, permitting long term therapy. In order to evaluate the clinical safety, the tolerability and the potential therapeutic efficacy of the HAT (Hoffmann-La Roche) a pilot study was prospectively performed in 5 pts with progressive, unresponsive A-GvHD. *Materials and Methods.* The criteria for inclusion in the study were the followings: 1) progression of A-GvHD after 3 days of therapy with 2 mg/kg methylprednisolone (MP) and CSA; 2) persistence of grade II A-GvHD after 14 days of therapy with MP+CSA; 3) persistence of grade III-IV A-GvHD after 7 days of therapy with MP+CSA. HAT was administered as single i.v. infusion of 1 mg/kg over 2 hours. In all cases the therapy with MP+CSA was maintained during HAT treatment. *Results.* Patient characteristics, response to therapy and outcome are reported in the Table:

				grade of A-GVHD			
UPN	age	DX	proph.	onset (organ)	pre-HAT (organ)	post-HAT (organ)	outcome (day)
237	17	ALL	CSA+MTX+PDN	I(S)	III(S.G.L.)	0	alive (284)
239	24	ALL	CSA+PDN	II(S.G.)	II(S.L.)	0	alive (270)
243	20	AML	CSA	III(S.L.)	IV(S.G.L.)	IV(G.L.)	dead (17)
244	17	SAA	CSA	I(S)	II(S)	0	alive (213)
184*	26	CML	NO	IV(S.G.L.)	IV(L)	IV(L)	dead (11)

*CML relapse treated by Buffy-coat infusion

No clinical side effect was observed during HAT infusion. At the last follow-up, 3 patients (UPN: 237-239-244) are complete responders, while 2 patients (UPN: 184-243) with grade IV A-GvHD died after 17 and 11 days from HAT administration. These deaths are not related to HAT. In patient n. 243 a resolution of skin envolvement with progression of gut and liver disease was observed. In conclusion the administration of HAT was safe and well-tolerated in all pts and had a clear anti-GvHD effect in 3 out of 5 pts.

079

THROMBOMODULIN AS A MARKER OF ENDOTHELIAL DAMAGE IN BONE MARROW TRANSPLANTED PATIENTS

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The veno-occlusive disease (VOD) of the liver is one of the early complications occurring after marrow transplantation and many data suggest that endothelial injury plays a pivotal role in its pathogenesis.
Since plasma thrombomodulin is thought to be of value as a marker of vascular endothelial cell membrane injury, we investigated its plasma concentration in bone marrow transplanted patients in order to better clarify the degree of the endotelial involvement.
The plasma thrombomodulin was monitored in 25 patients without thrombotic complications before transplant, on day 0 and weekly for one month thereafter and in 2 patients who developed VOD from day -7 to day +52. Plasma thrombomodulin resulted in the normal range in all the uncomplicated patients and in one patient with reversible VOD, while it was always very high in the second patient who developed a very severe VOD.
In conclusion, pre-transplant elevated levels of plasma thrombomodulin confirm the pathogenetic involvement of endothelial cells in VOD occurrence and suggest that the evaluation of this molecule may be of prognostic value.

080

ALLOGENEIC BONE MARROW TRANSPLANTATION (BMT) DURING THE CHRONIC PHASE OF CHRONIC MYELOID LEUKEMIA (CML). RESULTS FROM A SINGLE CENTRE DURING THE 1983-93 DECADE

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We have analyzed the results of treating 61 consecutive adult patients with CML in first chronic phase by high-dose therapy and BMT using fully HLA identical sibling donors (except for 3 cases of 1 antigen mismatch) during the period 1983-1993. Mean age was 35 yrs. Conditioning regimens were: total body irradiation (TBI), 10 Gy from a single C060 source in a single fraction at a low dose-rate (3.5-5 cGy/min) plus cyclophosphamide, 120 mg/kg (35 pts) until 1989 and Busulphan, 16 mg/kg plus cyclophosphamide 200 mg/kg (BU/CY) in 26 pts. GVHD prophylaxis also varied with time: CsA alone was used in 18 cases, T-depletion of the marrow with the Campath 1 monoclonal antibody was used in 7 cases and CsA plus a short course of 4 doses of methotrexate in 36 cases. All the T-depleted pts. belonged to the TBI group while most cases of CsA+MTX belonged to the BU/CY group (22 out of 26). Also the three 1 ag. mismatches belonged to the BU/CY group and received Csa+MTX. Thirty-four pts (55%) are alive, with a median survival of 72 mos (range 1-125). Of the TBI group, actuarial survival is 40% (95% C.I., 23-56%) at 10 yrs: in the BU/CY group, actuarial survival is 79% (95% C.I. 62-95%) at 4.5 yrs (p=0.02). The T-depleted pts did very poorly, with a high rate of rejection (28%) and relapse (100%). Excluding these patients, only two relapses occurred, one each in the TBI and BU/CY groups. Of the 21 pts who died in the TBI group, the main causes of death were the combination of GVHD, infection and interstitial pneumonitis while relapse was the cause in 3 pts. The main causes of death in the BU/CY group were GVHD/infection in 5 cases and VOD in one.
We confirm the low incidence of relapse after unmanipulated BMT and the high incidence after T-depletion. The good results obtained with the BU/CY combination might be due to the fact that pts so treated had an earlier BMT compared to those of the TBI group (interval from diagnosis to BMT 26±12 mos and 36±32 mos, respectively, p=0.05) and perhaps benefitted of a reduced transplant related toxicity in the most recent years. However, even if a retrospective comparison between the two regimens is not possible, BU/CY appears now as a good standard for BMT during the chronic phase of CML.

081

SURFACE RECEPTOR MOLECULES INVOLVED IN NK-MEDIATED LYSIS

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NK cells are a subset of CD3-16+56+ large granular lymphocytes which constitutively express cytolytic activity against susceptible tumor or virally infected cells. Although the mechanism(s) involved in tumor cell recognition and lysis are still poorly understood, a number of surface molecules which regulate the cytolytic activity and the activation of NK cells have been identified. The CD16 (Fcg-RIII) and CD2 molecules in humans and the NKRP-1 in rodents work as *triggering* surface receptors which deliver activatory signals while the CD94 (KP43) and *p58* molecules in humans and Ly 49 in mice display inhibitory functions. The latter molecules are involved in the recognition of specific MHC class I alleles which confer to target cells protection against the NK cell-mediated lysis. We have recently selected an anti-p58 mAb (CH-L) which reacts with a subset of NK and T cells.
Clonal analysis of the minor CD3+ p58+ T cell population revealed that, in most instances, these T cells display cytolytic *NK-like* activity. In addition, p58 molecules exerts inhibitory functions on the activation mediated by the CD3/TCR complex, in a similar manner as observed in the CD16-mediated activation of CD3-NK cells. Moreover, masking of p58 molecules by anti-p58 mAb may *restore* the susceptibility of cytolysis of tumor cells which express protective MHC alleles. These data suggest that p58 molecules may display a more general role of regulatory receptor in both NK and T cells with NK-like activity, by inhibiting activactory signals mediated by *triggering* receptors such as CD16 and the CD3/TCR complex.
The possible relevance of activatory and inhibitory NK receptor molecules in the development of new immuno-therapeutic strategies in leukemia will be discussed.

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REGULATION OF HEMATOPOIESIS BY HUMAN NATURAL KILLER CELLS

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NK cells are a subpopulation of lymphoid cells specialized in the killing of malignant or virus-infected cells in the absence of specific recognition and memory. Besides mediating cytotoxic functions, NK cells release a wide array of cytokines, some of which may play a role in the regulation of hematopoiesis. In our experiments we showed that human peripheral blood NK cells purified by negative selection released in short term unstimulated cultures both an erythroid burst promoting activity (BPA) and a myeloid colony stimulating activity (CSA).
Additional studies demonstrated that GM-CSF was the major, but not the only, component of the NK cell-derived CSA, which was also contributed for by IL3. Contact of purified NK cells with NK-sensitive, but not NK-resistant, targets led to the production of a myeloid colony inhiting activity (CIA), which was abrogated by treatment of the supernatants with a neutralizing anti-TNFα antiserum. These findings indicate that human NK cells are bidirectional regulators of hematopoiesis depending on their functional activation state and on the nature of the stimuli they receive. Although NK cells have T cell receptor and immunoglobulin genes in germ line configuration, allospecific NK cells do exist and specific recognition appears to require the expression of an autosomic recessive gene on target cells. These alloreactive NK cell clones specifically inhibit the *in vitro* growth of hemopoietic progenitors.
In conclusion, the complex role played by human NK cells in the regulation of hematopoiesis is characterized by both stimulation and inhibition of committed hemopoietic progenitor cell growth; furthermore, the latter phenomenon may take place in the setting of specific or non specific target cell recognition.

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CYTOTOXIC ACTIVITY OF LYMPHOCYTES STIMULATED BY HUMAN TUMOR CELLS TRANSDUCED WITH DIFFERENT CYTOKINE GENES

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Using different retroviral vectors a human lung tumor cell line (ADK-P) was transduced with the following cytokine genes: interleukin 2 and 7 (IL2, IL7), granulocyte macrophage-colony stimulating factor (GM-CSF) and tumor necrosis factor α (TNFα). Co-cultures were set up with cytokine tranduced tumor cells and allogeneic peripheral blood lymphomonocytes (PBL) at different ratios. After 7 and 28 days of co-culture the following aspects were evaluated: tumor growth capacity, lymphocyte phenotype, generation of specific and unspecific cytotoxicity, induction of mRNA for cytokines involved in tumor recognition and cytotoxicity.
After 7 days of co-culture in the presence of allogeneic PBL, the growth of GM-CSF and TNFα gene transduced tumor cells was reduced. PBL showed a CD3+ phenotype, a small degree of specific cytotoxic activity and a marked increase in IFNγ and TNFα mRNA expression. Because of the very limited number of PBL, the co-cultures could not be prolonged further.
After 7 and 28 days, PBL co-cultured with tumor cells transduced with the IL7 gene exerted an inhibitory effect on tumor cell growth. PBL showed a CD3+, CD4+ and CD56+ phenotype; after 7 days, the cytotoxic activity was unspecific while a certain degree of specific activity was observed after 28 days of co-culture. No differences in cytokine mRNA expression were seen in PBL stimulated with IL7 gene transduced tumor cells or with parental cells.
Tumor growth was partly reduced after 7 days and completely abrogated after 28 days by PBL co-cultured with tumor cells transduced with the IL2 gene. The PBL phenotype was as follows: 60% CD3+ cells with a CD4/CD8 ratio = 2/1 and 25% CD56+ after 7 days of co-culture, while after 28 days 95% of the population was represented by CD3+ cells with a CD4/CD8 ratio = 1/1.5, while the CD56+ cells were reduced to 4%. Largely unspecific cytotoxic activity was recorded after 7 days, while after 28 days an increase in specific cytotoxic recognition was observed; an enhanced TNFα mRNA espression was also seen.
These results suggest that different cytokine genes transduced into the DNA of the human tumor cell line ADK-P can stimulate allogenic PBL which in turn are capable of exerting a control of tumor growth through the amplification of the cytotoxic compartment. In this tumor model, the best anti-tumor activity has been obtained with neoplastic cells transduced with the IL2 gene.

084

CLINICAL RESULTS AND BIOLOGICAL MODIFICATIONS INDUCED BY rIL-2 IN AML PATIENTS TREATED IN DIFFERENT PHASES OF DISEASE

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Based on the results obtained *in vitro* and in animal models, 6 years ago we started a series of clinical studies utilizing IL-2 in patients with acute leukemias in a very advanced phase of disease. The first pilot study showed the feasibility of our high dose IL-2 schedule, which consisted of a 5-day cycle to be repeated 4 times with a 72-hour rest between each cycle, at escalating doses administered i.v. in continuous infusion up to the maximum tolerated dose. We also observed evidence of a potential clinical activity in a particular subgroup of patients with AML with limited bone marrow blastosis (>5% to <30%); particularly, 1 pt achieved a CR which lasts from >6 years. To confirm the above we planned a second pilot study in patients with refractory or relapsed AML, not eligible for conventional treatments, with a detectable bone marrow blastosis not exceeding 30%. The IL-2 schedule utilized was similar to the one described above with doses escalating from 8 up to 18×10^6 IU/sqm/day. Five out of 9 treated patients achieved CR and started a maintenance treatment with a lower dose ($4\text{-}8\times10^6$ IU/sqm/day) administered subcutaneously for 5 days each month. One patient relapsed and 4 are in CCR at 21, 38, 40 and 40 months.
These encouraging clinical results prompted us to plan an Italian multicentric prospective randomized trial, coordinated by the GIMEMA group, to evaluate the efficacy of IL-2 administered as post-remissional treatment in AML patients achieving a second or subsequent CR and not eligible for transplant procedures. This study is ongoing and may contribute to clarify the clinical impact of IL-2 in the treatment of AML.
Biological monitoring of patients treated with IL-2 always showed marked phenotypic and functional modifications of the immune system: increased CD3+ cells, both circulating and in the bone marrow; amplification of the "cytotoxic" cells (CD16+, CD56+); enhancement of NK activity as well as of IL2-induced LAK activity; moreover, IL-2 is capable of generating *in vivo* a proportion of endogenous LAK cells which are rarely found under basal condition. IL-2 infusion is also associated with the *in vivo* detection of circulating levels of TNF and γ-IFN. These phenotypic and functional modifications appear to occur in all treated patients and their relationship with the heterogeneous clinical response remain to be clarified.

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GRAFT vs LEUKEMIA EFFECT IN ALLOGENEIC BONE MARROW TRANSPLANTATION

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We have analyzed the effect of different GvHD prophylaxis protocols on the occurrence of leukemia relapse following allogeneic bone marrow transplantation (BMT) in patients with chronic myeloid leukemia (CML) and in patients with acute myeloid leukemia (AML).
In patients with chronic myeloid leukemia the use of cyclosporine A (CyA) 2 mg/kg/day pre-transplant improves tolerance to the transplant, but is associated with a higher relapse rate. The same can be said of T cell depletion. GvHD prophylaxis is the single most significant prognostic factor for relapse in multivariate analysis.
In patients with acute non lymphoid leukemia (ANLL) we have shown that the use of high dose cyclosporin A (CyA) in the first 10 days post-BMT produces a significant increase of leukemia relapse. We have confirmed this data in a large number of ANLL patients and with multivariate analysis: GvHD prophylaxis is the single most relevant factor predicting disease free survival.
These observations suggests that with a given conditioning regimen (Cyclo+TBI) there is a relevant impact of *immune control of leukemia* and events crucial for long term control occur between day –7 and day +10 of transplant.

086

IMMUNOLOGIC AND CLINICAL MODIFICATIONS FOLLOWING LOW DOSES SUBCUTANEOUS ADMINISTRATION OF rIL2 IN NON-HODGKIN'S LYMPHOMA PATIENTS AFTER AUTOLOGOUS BONE MARROW TRANSPLANTATION

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Seven patients with high-grade non-hodgkin's lymphoma (HG-NHL) started treatment with recombinant interleukin 2 (rIL2) within 2 months by the autologous bone marrow transplantation (ABMT). rIL2 was administered subcutaneously (s.c.) at a dose of 2 international megaunit (IMU)/m^2 every other day for 2 weeks and then 4 IMU/m^2 twice a week for one year. Immunological studies, including T and natural killer (NK) cell subsets assessment together with functional assays, such as NK activity and reverse adcc, were performed before therapy, after 2 weeks and then monthly. Phenotypic analysis showed a significant increase (p=0.01) of CD16 and CD56 NK cells, from 13 to 36% and from 16 to 45%, respectively. In particular, CD56 bright NK cell population showed a 10-fold increase, while CD56dim NK cells remained unmodified compared with pre-treatment values. The expression of IL-2 receptors was also studied and a significant increase (p=0.01) of CD122 (p75) positive cells from 12 to 38% was found, while no significant increase was observed in CD25 (p55) positive cells. Furthermore, rIL2 administration led to an increase of NK activity even at the lowest effectors: target ratio and to an increase of CD16 reverse ADCC. These phenotypic and functional modifications lasted throughout the duration of rIL2 therapy, also after longtime by the rIL2 administration. Clinical tolerance was good, fever and moderate fluid retention were observed only at the onset of rIL2 therapy in 3 cases. None of the patients have progressed 5, 8, 9, 12, 13, 14 and 24 months respectively after starting therapy. Interestingly, one patients with hepatic residual disease after ABMT obtained complete remission after 10 months of rIL2 treatment.
In conclusion, our preliminary data suggest that infusion of rIL2 s.c. after ABMT is safe, can selectively increase NK cell number and function and may result in a beneficial effect on the minimal residual disease.

087

IN VITRO SUSCEPTIBILITY OF ACUTE LEUKEMIA CELLS TO THE CYTOTOXIC ACTIVITY OF ALLOGENEIC AND AUTOLOGOUS LAK EFFECTORS: CORRELATION WITH THE RATE AND DURATION OF THE COMPLETE REMISSION AND SURVIVAL

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Acute lymphoblastic leukemia (ALL) and acute myeloblastic leukemia (AML) blasts were studied for their sensitivity to the lytic activity of normal allogeneic interleukin-2 (IL2) activated killer (LAK) cells, and of autologous LAK effectors generated at the time of complete remission (CR). In 12 of 23 ALL cases (52%) the blasts were susceptible to normal LAK cells (>15% lysis) and 10 of them achieved a CR. Of the remaining 11 LAK resistant cases, 7 obtained a CR. No correlation was found between susceptibility to LAK activity, cytomorphology, immunophenotype, CR duration and survival.
Eighteen of the 26 AMLs tested (70%) were susceptible to normal LAK cells, and 9 of the 13 cases studied (70%) were also lysed by autologous LAK effectors generated at CR. No clearcut correlation was observed between blast sensitivity to normal LAK cells and morphological cytotype, though a higher incidence of resistant cases was observed in the M4 subgroup. All AML cases susceptible to normal LAK cells but one achieved a CR, while this occurred only in 3 of the 8 resistant cases ($p = 0.004$). The median survival and event-free survival duration in the resistant patients were significantly shorter ($p = 0.03$ and $p = 0.02$, respectively) compared to those of the susceptible patients.
Taken together, these findings suggst that in AML, and not in ALL, the LAK cell phenomenon may be predictive of clinical course and overall outcome of the patients.

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ANTITUMOR ACTIVITY OF BER-H2/SO6 IMMUNOTOXIN IN VITRO AND IN SCID MICE XENOGRAFTED WITH HUMAN CD30(Ki-1)+ ANAPLASTIC LARGE CELL LYMPHOMA

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Ki-1 (CD30)+ anaplastic large cell lymphoma (ALCL) is a quite distinctive clinicopathologic entity which has been recently integrated into the updated Kiel classification as a high-grade lymphoma. To develop a novel adjunctive therapy, we evaluated the antitumor activity on ALCL cells of an immunotoxin (IT) constructed by coupling the plant emitoxin Saporin (SO6) to the Ber-H2 monoclonal antibody (mAb) directed against the CD30 molecule, a newly recognized member of the TNF/NGF receptor superfamily. Tests were performed *in vitro* against the CD30+ ALCL-derived cell line JB6 and *in vivo* using our newly established SCID mouse model of human xenografted CD30+ ALCL. In vitro, Ber-H2/SO6 was selectively and highly toxic to the JB6 cell line, with an IC50 of 5×10^{-12} M as SO6, far below the toxicity of irrelevant IT or the toxicity to irrelevant target. In vivo, a 3-day treatment with non toxic doses of Ber-H2/SO6 IT, corresponding to 50% of the LD50, induced lasting complete remissions in 80% of mice starting the treatment 24 h after tumor transplantation. When injected at later stages of tumor growth (mice bearing subcutaneous ALCL tumors of 40 to 60 mm^3 volume), Ber-H2/SO6 induced CR in 6 of 21 mice and, as shown in Table below, significantly delayed tumor growth rate ($p<0.01$). In contrast, neither the unconjugated mAb nor the toxin alone had any antitumor effect.

Groups	*Days to 500 mm^3*	*Tumor growth delay (days)*
Controls	18.28±0.99 (n=19)	
Ber-H2/SO6 IT	39.98±5.85 (n=21)	21.7±4.7
SO6	23.26±5.05 (n=7)	5.0±3.8
Ber-H2	17.97±0.67 (n=7)	

We conclude that Ber-H2/SO6 IT is an effective agent against CD30+ ALCL growing in SCID mice, suggesting a possible therapeutic role in patients with CD30-expressing neoplasms refractory to conventional therapy.

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IN VITRO INDUCTION OF α-IFN STIMULATED GENE EXPRESSION (TYk2, ISGF3) IN CELLS OF PATIENTS WITH CHRONIC MYELOGENOUS LEUKEMIA (CML)

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Very few data are available on the molecular mechanism of α-IFN responsiveness of CML cells. The immediate cellular response to IFN-α is activation of a set of new proteins necessary for the anti-proliferative effects of the cytokine. The biological response to interferon (α-IFN) is initiated by cell surface receptor binding and relies on induction of a set of IFN-α specific proteins, coding from genes under transcriptional control.
The control of expression of these genes is mediated through a genetic element (named IFN-α-stimulated response element or ISRE) located in their promoter regions, through the action of a sequence specific DNA binding protein termed IFN stimulated gene factor 3 (ISGF3). ISGF3 is composed of four proteins, a DNA binding protein termed ISGF3-γ and three proteins which together form ISGF3-α. IFNα activation assemble the ISGF3-α proteins (by a phosphorylation mediated by a tyrosine kinase protein named Tyk2, on tyrosine residues, by nuclear translocation of the proteins, and their transcriptional activation).
By use of a quantitative polymerase chain reaction (RT-PCR), and using a set of sequence specific primers for Tyk2, and for ISGF3 genes, we assessed the levels of expression before and after α-IFN exposition of freshly isolated cells from 5 patients affected by CML, collected at the diagnosis time.
Furthermore, we correlate the expression of these ISGF3 genes with the α-IFN induction of apoptosis assessed by an agarose-gel-fragmentation-assay.
In all the samples, 36 hours after the exposition of the cells at the immune-modulating agent, we found a marked and increased expression of the levels of these IFN related-genes in all the patients with CML. Induction of apoptotic pattern of gel fragmentation by IFN exposition was reduced in all the CML samples analysed, compared with exposition of the same cells with other immuno-modulating agents alone or in association (IL2, IL4) Our preliminary study, confirm the importance of the ISGF3 genes pattern of expression in CML patients exposed to an IFN therapy, suggesting a possible role of these genes on *different responsiveness in vivo* to the IFN treatment.

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STROMAL CELLS FROM LYMPHOID TISSUE: PHENOTYPE AND CYTOKINE PRODUCTION ANALYSES

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Lymphoid tissues contain several non-lymphoid cell types, such as endothelial and dendritic cells, that contribute to lymphoid microenvironment. Hystological analysis of lymph nodes (LN) described in the past an additional cell type in LN referred as fibroblastic reticular cells or stromal cells that interact with recirculating lymphocytes and may contribute to the structure and function of lymphoid organs. Very little in present in the literature about the phenotype and function of these cells. In this study we present data showing *in vitro* expansion of stromal cells from human tonsils (HTSC) with analysis of surface markers and cytokine production.
Primary cultures of HTSC were obtained following enzymatic treatment of tonsils from young individuals and expanded *in vitro* for five passages. Phenotype and cytokine production was analyzed on resting HTSC or after activation with IFNγ (100 U/mL) and/or TNFα (500 U/mL). Cells used for FACS analysis were harvested by trypsinization, let recover overnight on a rocker platform in the incubator followed by labelling with a panel of mAb. Cytokine production was analyzed by ELISA tests of the supernatants of the cultures collected at different time points (24, 48, 72, 96 hours) and by analysis of mRNA expression by RT-PCR.
Surface marker analysis showed that resting HTSC express ICAM-1 (27%), VCAM-1 (5%), CD29 (70%), CD44 (85%), CD49a (30%), CD49b (60%), CD49c (10%), CD49e (21%), CD51 (20%), CD61 (7%) but are negative for HLA-DR, CD4, CD14 and other myeloid or lympoid markers. Treatment with IFNγ and TNFα increased the expression of ICAM-1 (80%) and HLA-DR (90%). Factor VIII and cytokeratins were not expressed by HTSC suggesting that HTSC were not derived from endothelial or epithelial cells. Cytokine production assays indicated that IL2 and IL4 were not produced by HTSC while IL6 production was costitutive and GM-CSF production could be induced by HTSC treatment with TNFα.
These results show that HTSC is a distinct cell type present in lymphoid tissues that can be distinguished, phenotipically, from other non-lymphoid cell types present in lymph nodes such as endothelial and dendritic cells. Stromal cells may play an important role in the microenvironment of lymphoid tissues through their production of soluble factors and direct cell-cell interaction and, to this respect, the HTSC model system will be useful for *in vitro* study at the cellular and molecular level of the relationships between the stromal and lymphoid cells.

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IN VITRO PREDNISONE RESISTANCE IN ADULT ACUTE LYMPHOBLASTIC LEUKEMIA

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The efficacy of glucocorticoids in lymphoprolipherative disorders has been well established. It has recently been reported that the clinical outcome of childhood acute lymphoblastic leukemia (ALL) is strictly related to *in vitro* prednisone resistance displayed by blast cells.
This study aimed to investigate the biological significance of prednisone resistance in adult ALL. Samples from 20 ALL patients were exposed to prednisone (20 ng/mL-10 mg/mL) for 48 hours, and drug sensitivity was evaluated by the XTT-PMS colorimetric assay. Both the IC10 and the IC50 varied greatly among the samples (IC10 range: <10 μg/mL-210 μg/mL; IC50 range: 250 μg/mL->10 mg/mL), and the average IC50 was higher than that observed in childhood ALL samples.
We have also evaluated prednisone-induced programmed cell death using a cytofluorimetric method that allowed us to quantitate the percentage of apoptotic cells in a given sample. Prednisone was able to induce apoptosis even in samples that were scarcely sensitive to the drug. The expression of the BCL2, instead, seemed to be related to prednisone resistance. Clinical correlations will be provided.

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DIFFERENTIAL RESPONSES OF MYELOID PROGENITOR CELLS FROM PATIENTS WITH MYELODYSPLASIA TO THE EFFECTS ON MULTIPLE CYTOKINE COMBINATIONS IN VITRO

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Myelodysplastic syndromes (MDS) comprise a heterogenous group of multipotent stem cell disorders characterized by qualitative maturation defects and a frequent progression to leukemic transformation. A combination of growth factors, necessarily including stem cell factor, has shown to induce proliferation and differentiation of early bone marrow progenitor cells *in vitro*.
Light density bone marrow cells of 30 MDS patients of different FAB subtypes were grown in stroma-free liquid cultures for 4 weeks in 25 cm^2 tissue cultue flasks with the addition of SCF, IL-3, IL-6 and GM-CSF every 48 hours. At weekly intervals, clonogenic activity, cell morphology and karyotype (analyzed both with G-banding on metaphases or with interphase FISH) were evaluated in the cultures.
Whn cells were studied for the *in vitro* effect of the cytokine combination a marked heterogeneity was detected in each FAB categories, but 4 different patterns emerged. One pattern was consistent with maturation of normal progenitor cells; a second pattern indicated maturation of cytokine responsive MDS progenitor cells; a third pattern showed complete unresponsiveness to growth factors, while a forth pattern was consistent with stimulation of leukemic cell growth.
These results suggests that responsiveness of myeloid progenitor cells in MDS is highly heterogenous; however this system may provide useful informations on the control of leukemic cell growth and on disease progression.

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EXPRESSION AND STIMULATORY ACTIVITY OF INTERLEUKIN-9 (IL-9) IN HUMAN MYELOID LEUKEMIC CELLS

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In this study, we evaluated the proliferation of 3 leukemic cell lines and 14 primary samples from acute myeloblastic leukemia (AML) patients in response to rh-IL-9 alone and combined with rh-IL-3, GM-CSF and SCF (c-kit ligand). The colony forming ability of HL 60, K562, KG1 cells and 7 fresh AML cell populations was assessed by a clonogenic assay in methylcellulose. In addition, in 7 AML cases the number of S-phase cells induced by IL-9 was determined, after 3 days of liquid culture, by the bromodeoxyuridine (BRDU) incorporation assay.
IL-9, as single cytokine, stimulated at various concentrations the colony formation of the 3 myeloid cell lines under serum-containing and serum-free conditions. Moreover, anti-IL-9 monoclonal antibodies (MoAbs) completely abrogated the proliferative response of leukemic cells to exogenous IL-9 and reduced the spontaneous growth of 2 cell lines cultured in absence of serum. When tested on fresh AML samples, optimal concentrations of IL-9 resulted in the increase of the blast colony formation in 7 out of 7 cases (22±11 SEM colony-forming unit-leukemic-CFU-L/10^5 cells plated in control cultures compared to 113±37 SEM in IL-9 supplemented dishes; $p< 0.05$). IL-9 stimulated 34.2% of CFU-L induced by phytohemagglutinin-lymphocyte conditioned medium (PHA-LCM) and it was the most effective CSF for promoting leukemic cell growth among those tested in this study (i.e. SCF, IL-3, GM-CSF). Moreover, IL-9 alone augmented significantly the number of leukemic cells in S-phase in 5 out of 7 cases.
The addition of SCF to IL-9 demonstrated an additive or synergistic effect of the 2 cytokines in 5 out of 7 AML cases tested for their CFU-L growth. The same combination resulted in a higher DNA synthesis rate in 2 leukemic samples. Positive interaction was also observed when IL-9 was combined with IL-3 and GM-CSF (5 and 9 out of 14 cases, respectively). Finally, reverse transcriptase-polymerase chain reaction amplification (RT-PCR) demonstrated the constitutive expression of IL-9 mRNA in 9 out 12 AML samples studied. Moreover, secreted IL-9 was found in the supernatant of AML samples.
In summary, our results indicate that IL-9 is expressed in AML cells and it may play a role in the regulation of the proliferation of myeloid leukemic cells.

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CYTOLOGIC AND CYTOGENETIC PROGRESSION OF B-CELL CHRONIC LYMPHOCYTIC LEUKEMIA

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Different evolution patterns of B-cell chronic lymphocytic leukemia (B-CLL) have been described, based on sequential morphologic and immunologic studies. More recently, introduction of cytogenetic analysis as part of the routine laboratory workup in B-CLL and the identification of three distinct related forms, namely typical CLL, with less than 10% large lymphocytes (LL) and prolymphocytes (PL), mixed CLL with 10-55% LL and PL, and prolymphocytic leukemia (PLL) with more than 55% PL, prompted investigators to perform correlation studies of cytologic and cytogenetic features during the natural history of this lymphoproliferative disorder.
Evidence is accumulating that, among unselected patients presenting with B-CLL and related disorders, a progressive increase of LL and PL occurs more frequently in patients with trisomy 12 and t(11;14) than in patients with other chromosome aberrations or normal karyotype.
Patients with trisomy 12 usually present with the features of typical CLL and may evolve into mixed CLL. The immunophenotype in these cases does not appear to differ significantly as compared with that of other typical CLL cases, with CD5/CD23 positivity and weak surface immunoglobulin expression (sIg). Cytogenetic evolution have been detected in some of these cases.
Patients with the t(11;14) may show overlapping features with intermediate lymphocytic lymphoma (ILL) in leukemic phase.
Those patients with the t(11;14) and morphology conforming to the FAB criteria for the dignosis of mixed CLL may represent a distinct subset of B-CLL and related disorders in which progressive accumulation of prolymphocytes as well as karyotype evolution may be seen in the majority of cases. Unlike most cases of CLL, immunologic findings in this cytogenetic subset of CLL and related disorders almost invariably show a bright sIg staining pattern, frequent CD22 and FMC-7 positivity, incostant CD5 and CD23 positivity.
Morphologic, immunologic and cytogenetic studies in B-CLL and related disorders may be of value for the identification of disease entities characterized by distinct evolution patterns, possibly requiring different therapeutic approaches.

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p53 GENE MUTATIONS ARE ASSOCIATED WITH ADVANCED FORMS OF MULTIPLE MYELOMA

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Mutations affecting the p53 tumor suppressor gene have been found associated with several types of human cancer. Loss of the normal growth inhibitor activity of p53 gene is achieved in most of the tumors through a point mutation of one p53 allele and deletion of the other p53 allele. Mutations of p53 are generally found in exons 5 to 9 and are clustered in four regions of the protein which are highly conserved through the evolution. In the context of lymphoid malignancies p53 mutations are associated at appreciable frequency with Burkitt lymphoma, and B-chronic lymphocytic leukemia. Mutations were not found at significant frequency in non-Hodgkin lymphoma, acute lymphoblastic leukemia, peripheral T-cell lymphoma, and hairy cell leukemia. We have investigated the frequency and type of p53 mutations in a panel of 52 cases of multiple myeloma (MM) representative of the different phases of the disease (indolent: 12; chronic: 24; acute-leukemic: 16). DNAs were analyzed for mutations in exons 5 to 9 of p53 gene by polymerase chain reaction (PCR), single strand conformation polymorphism (SSCP), and direct sequencing of PCR-amplified fragments. p53 mutations were detected in seven cases (7/52-13%) and occured within highly conserved domains of p53 coding sequence: five cases involved exon 8 (codons 272,275,282,284,286) and two cases exon 6 (codon 193) and 7 (codon 248), respectively. Mutations were represented by a single nucleotide change with a missense mutation resulting in amino acid substitution. All the mutated cases belong to the acute-leukemic phase of MM (7/16-43%). Interestingly three of these cases were analyzed at presentation (indolent or chronic phase) and were found negative for p53 mutation. Tumor progression is thought to be the result of a sequential accumulation of genetic lesions. The results reported here support the notion that development of MM is a multistep process and strongly suggest that the occurence of p53 mutations may represent one important late event in tumor progression of MM.

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MULTIPLE MYELOMA: SURVIVAL ANALYSIS CONFIRMS THE PROGNOSTIC SIGNIFICANCE OF BONE MARROW PLASMA CELL PHENOTYPE

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We analyzed the immunological phenotype of bone marrow (BM) plasma cells from 104 monoclonal gammopathies in order to verify its impact in discriminating between multiple myeloma (MM) and monoclonal gammopathy of undetermined significance (MGUS), and in predicting survival. No statistically significant difference was observed between these two groups in the expression of the adhesion molecules CD11a, CD18, CD44, CD54. Of interest, CD56 was not only expressed by myeloma cells, but by all monoclonal plasma cells, either in MM or MGUS. The only difference between the two groups was the percentage of plasma cells CD56+, depending on the ratio between normal and abnormal plasma cells.
In this study we confirm by a survival analysis our previous observation on the prognostic importance of the expression of surface immunoglobulins by plasma cells: patients with "early-PC" (immature plasma cells coexpressing plasma cell antigens and surface immunoglobulins) (33/65) have a median survival of 39 months, whereas patients with late-PC (typical mature cells expressing plasma cell markers only) (32/65) have not reached median survival at 45 months ($p<0.02$).
The same phenotypic characteristic has been observed in 30% of MGUS patients. 12/39 showed the early-PC pattern on BM plasma cells. During a median period of observation of 26 months, 3 out of 12 early-PC patients (25%) underwent malignant transformation, while only 1 out of the 27 late-PC patients (3.7%) developed MM ($p<0.04$).
We can therefore confirm the prognostic significance of the phenotypic analysis of BM plasma cells, that can predict survival in myeloma patients and malignant transformation in MGUS.

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MOLECULAR MECHANISMS OF TUMOR PROGRESSION IN BCR/ABL+ AND BCR/ABL– CHRONIC MYELOPROLIFERATIVE DISORDERS

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Chronic myeloproliferative disorders (CMPD) may be distinguished into two main groups depending on the presence or absence of the *BCR/ABL* rearrangement. BCR/ABL^+ CMPD are represented by chronic myeloid leukemia (CML), whereas BCR/ABL^- CMPD include myelosclerosis with myeloid metaplasia (MMM), polycythemia vera (PV), essential thrombocythemia (ET), juvenile chronic myeloid leukemia (JCML), as well as the so called BCR/ABL^- CML. BCR/ABL^+ CMPD evolve into an acute phase in virtually 100% of the cases, whereas the acute evolution of BCR/ABL^- CMPD is restricted to 5-20% of the cases, depending on the type of disease. The molecular pathology of tumor progression in CMPD is unknown. We have investigated the involvement of tumor suppressor genes (p53 and RB1) and dominantly acting oncogenes (N-, K-, H-Ras genes) in both BCR/ABL^+ and BCR/ABL^- CMPD at different stages of the disease, including 26 cases of CML blast crisis (17 myeloid, 8 lymphoid, and 1 megakaryoblastic blast crisis), 10 MMM, 6 PV, 12 ET, 8 BCR/ABL^- CML, and 1 JCML. The presence of mutations in p53 exons 5 through 9, as well as in RB1 exons 10-27 and in N-, K-, H-Ras exons 1 and 2 was tested by the PCR-single strand conformation polymorphism technique and by PCR direct sequencing. In addition, Southern blot analysis was used to investigate the occurrence of gross rearrangements in the p53 gene as well as loss of heterozygosity at 17p13, the site of p53. Acute phase BCR/ABL^- CMPD displayed a high frequency of p53 (2/7) and Ras (3/7) lesions, whereas BCR/ABL^- CMPD in chronic phase displayed only germline p53 and Ras sequences. Among the different types of BCR/ABL^- CMPD, p53 mutations were found in 1/3 acute phase MMM and in 1/4 acute phase BCR/ABL^- CML, whereas Ras mutations were detected in 2/3 cases of acute phase MMM and in 1/4 case of BCR/ABL^- CML. The occurrence of p53 and Ras family genes mutations in acute phase ET and PV is currently under study. In contrast to the relatively high frequency of p53 and Ras mutations in acute phase BCR/ABL^- CMPD, p53 inactivation was restricted to 1/26 cases of BCR/ABL^+ CML blast crisis and Ras activation was not detected in any of these cases. No alterations of the RB1 gene were detected in any sample of either BCR/ABL^+ or BCR/ABL^- CMPD. Altogether, our data suggest that p53 inactivation and/or Ras mutations might play a role in the progression from chronic to acute phase of BCR/ABL^- CMPD. Conversely, among BCR/ABL^+ CMPD, neither p53 nor RAS genetic lesions play a significant role in tumor progression, namely in the evolution into BCR/ABL^+ CML blast crisis. The low frequency of p53 mutations in BCR/ABL^+ CML blast crisis is intriguing, since i(17q), leading to the loss of one p53 allele, is a recurrent cytogenetic lesion in these tumors. It is possible that a tumor suppressor gene other than p53 and mapping to 17p is involved in BCR/ABL^+ blast crisis, as it has also been suggested in the case of gliomas and medulloblastomas.

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STRESS INFLUENCES THE PROGRESSION AND RESPONSE TO CHEMOTHERAPY OF TLX5 LYMPHOMA IN MICE

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The possibility that psycho-social factors might influence the incidence and progression of cancer is a challenging hypothesis, still awaiting conclusive scientific proof. Central nervous system has been shown to influence immunitary functions via neuroendocrine circuits. Specific psychological modalities of adaptation to cancer diagnosis have been shown to correlate with better outcome (fighting spirit) or worse survival (hopelessness-helplessness) in patients with mammary carcinoma or Hodgkin lymphoma. When experimental stressors were applied to mice bearing solid malignant tumors, tumor progression and in particular metastasis were significantly modified by stress. The response to chemotherapy with Cyclophosphamide or razoxane was similarly reduced by the stressors.
The aim of this communication is to report the results obtained applying rotational stress (RS) to CBA mice bearing TLX5 lymphoma. The application of RS to mice of both sexes inoculated with 10^2-10^5 tumor cells is devoid of significant effects. On the contrary, in female mice implanted with 10 tumor cells, RS significantly increases lymphoma progression in terms of tumor takes and survival time; no significant effect is observed in male mice also with this reduced tumor inoculum size. The treatment with CCNU cures 5 out of 10 non-stressed mice ($p<0.001$); upon application of RS, no cure is observed with a mean survival time of 18.6 days in comparison with 24.2 days for non stressed treated mice (Kaplan Meier analysis, log-rank test, $p<0.001$).
These results indicate that the application of an experimental stressor is capable to reduce the survival time of mice implanted with TLX5 lymphoma. This reduction is significant only with a limited tumour burden and occurs only in female mice. Moreover, this effect is more pronounced in January as compared with June. The seasonal dependency of the effects of RS is in agreement with the results obtained in CBA mice bearing MCa mammary carcinoma. These data could be interpreted assuming the occurrence of circa-annual rhythmic variations in endocrine functions mediating effects of stress on immune or NK anti-tumor responses of the host. At the same time, these results indicate that the same anti-tumor or NK functions of the host participate in determining the success of antitumor chemotherapy, being at the same time amenable to modulation by stress. Further research is in progress, aiming to identify the nature of the neuro-endocrine and immune factors involved in the reported effects of experimental stressors. These results seem of interest for both their experimental and clinical implications.

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MECHANISMS OF CELL CYCLE CONTROL IN NORMAL AND MALIGNANT HEMOPOIESIS: CYCLINS AND CYCLIN-DEPENDENT KINASES

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Recent studies into molecolar process that regulates cell proliferation have demonstrated a central role played by a family of proteins, the cyclins, and their catalitic subunits, the cyclin-dependent protein kinases (CDKs). Aim of our study was to investigate in normal and neoplastic human hemopoietic models, as well as in samples from patients with hemopoietic malignancies, the role of two proteins, cyclin A and B, and their catalitic subunits, CDK2 and CDC2. Distribution in the different cell cycle phases, as low proliferative-low RNA (G0) cells, G1, S and G2M was established by DNA/RNA flow cytometry (acridine-orange) and then correlated with protein expression tested by Western blot. Molecular changes occuring in normal human hemopoietic cells during recruitment into the cell cycle were measured at different time points using T-lymphocytes activated by anti CD3 and IL-2. Unstimulated lymphocytes (t=0) after monocyte depletion were 98.4% in G0 and were characterized by absence of cyclin A and B expression. CDK2 and CDC2 were strongly positive after 18 hours, when 25.1% of cells reached G1, although a lower protein expression was also found in unstimulated G0 lymphocytes. At 18-24 hours before cells entering into S phase we start to detect expression of cyclin A and cyclin B wich continue to increase until 48 hours (S=14.1%) Evaluation of protein expression in hematologic cell lines (U937, K562, KG1, U266, Mo7, Daudi) showed always a constant positivity of CDK2, cyclin A, Cyclin B and CDC2. Protein positivity was correlated with absence of cells in G0 but was independent from the origin of cell line analyzed (myeloid, lymphoid leukemia, as well as myeloma cells). Preliminar evaluation of cyclins and their catalitic subunits in leukemic patients showed in ALL higher expression of cyclin A and CDK2 which correlates with high S-phase values. In the other samples cell cycle distribution as well as expression of cyclins, was heterogeneous.
Our study demonstrates the role of cyclins in human proliferating cells, by absence of expression of cyclin A and B in resting T-lymphocytes and by their presence in all leukemic cell lines tested. The role of such proteins in hematologic neoplastic disease remaine to be defined.

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VALUTATION OF BAX GENE EXPRESSION, AN APOPTOSIS-RELATED GENE, IN PATIENTS AFFECTED BY HEMATOLOGIC DISEASES: A PRELIMINARY DATA

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Bcl2 protein is able to repress a number of apoptotic death programs, and this mechanism is mediated by interaction with other proteins. Recently, some studies have identified a Bcl2-associated protein of 21Kd molecular weight, named Bax. The corresponding gene is composed by six exons and shows a complex pattern of alternative RNA splicing, which gives rise to three cytosolic proteins named α, β, γ sub-unit, respectively. Bcl2 *in vivo* forms heterodimers with Bax homodimers. Overexpression of Bax proteins accelerates the apoptotic death induced by different stimuli, so that, the Bcl2/Bax ratio determines survival or death after an apoptotic stimulus.
So far, few data are available about the expression of Bax gene in hematologic diseases, in particularly in non Hodgkin lymphomas (NHL). We studied the presence and level of transcript in different lymphomas and during different phases of the disease, by an RT-PCR approach.
We have extracted RNA from normal supjects, patients with CML, and with low grade NHL. All the patient were studied for the immuno-phenotype expression.
Using a set of sequence specific primers, we were able to distinguish the differen ratio of expression between α, γ and β sub-units of Bax gene. When α, β, or γ transcripts were present an amplified fragment of 227bp was obtained, while only a fragment of 339bp was amplified when the α and β transcripts were expressed.
We have demonstrated the presence of the expression of $\alpha\beta$ Bax genes in all the samples analyzed. In all the B-NHL and B-CLL we were able to detect a discrete amount of gene expression. We also compared the levels of expression of $\alpha\beta$ Bax genes in NHL and in CML. Our preliminary result indicates that high level of these transcripts are present in both these hematologic disease. We are now studing the ratio of different Bax gene subunit, during the phase of the disease. Control of the Bcl2-Bax ratio may provide more light on apoptosis induction of different cell types, and also on the mechanism of chemotherapy employing purine analogs as apoptotic inducers agents.

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IDENTIFICATION OF THE UROKINASE-TYPE PLASMINOGEN ACTIVATOR RECEPTOR IN AML BLASTS

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The aim of this study was to investigate by flow cytometry the expression of the UPA-receptor (urokinase type plasminogen activator - CD87) on the blastic population of AML and ALL patients in order to evaluate whether the presence of this molecule could be associated with peculiar clinical and biologic features of leukemic cells. The mean percentage of positive cells and the molecular equivalents of soluble fluorochrome (MESF) were calculated in 8 AML (6: M4; 1: M1; 1: M0, according with FAB classification) 3 ALL and 4 healthy subjects who served as controls. In addition cell reactivity was evaluated in peripheral blood lymphocytes, monocytes and neutrophils derived from either normal and leukemic samples. Five different monoclonal antibodies (MAbs) (clones: 3B10; VIM5; 109; 68; 100) were used in order to detect the distinct forms of this cellular receptor. Cell reactivity varied significantly from case to case, depending also on the MAb used for the flow cytometry analysis. In brief, 3B10 and VIM5 MAbs were found to be positive in more than 90% of monocytes and neutrophils, while the number of positive cells was decreased (60%) using the 109 MAb. However, either 68 and 100 MAbs recognized only a low number of blood monocytes and neutrophils (8-20%). Lymphocytes were unreactive with all the five UPA-R MAbs. ALL cells were found to be CD87 negative in all cases. On the contrary, blasts from AML showed a heterogeneous pattern of expression for the UPA-R MAbs, being reactivity strictly dependent on the MAb used, and, in a higher extent, on the degree and type of maturation of the blastic cells. The number of blasts recognizing 3B10 and VIM5 MAbs was significantly higher (mean percentage 26,6 27,7-SD, and 25,9 29,7 SD, respectively) than that reacting with the remaining MAbs (109 MAb: 10,4 7,9; 68 MAb: 4,1 4,0; 100 MAb: 5,7 6,3), irrespective of the FAB subtype. Since proteolytic enzymes, like UPA, play a key role in the dissolution of the extracellular matrix, and therefore in the cell egress from bone marrow through the physiological tissue barrier, it is conceivable that the expression of the UPA-R could contribute to the invasive properties and possibly metastatic potential of leukemic cells.

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MORPHO-IMMUNOLOGICAL AND CLUSTER ANALYSIS APPROACH FOR THE CHARACTERISATION OF THE BLASTIC POPULATION IN ACUTE MYELOID LEUKEMIA

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The necessity of a cytofluorimetric analysis based on the definition of clusters of cells with similar morphological patterns and immunological properties was pointed out in several recent works. For a correct application of this approach a careful and precise standardisation of the analysis methodologies is mandatory. By applying standard single and double fluorescence flow cytometry technique and following a careful standardisation of the Instruments (FACScan and FACStar Plus, Becton Dickinson) with FITC calibrating microbeads, we studied the immunophenotype of 65 AML at presentation and at relapse in order to correlate the morpho-immunological patterns of the blasts to the FAB classification. As far as light scattering properties is concerned, all the identifiable subpopulations were gated and then analysed in four quadrants multicolour dot plot statistic evaluating SSC and FL1 or FL2 for FITC and PE Moabs respectively. As far as CDs intensity of expression is concerned, the mean channels of fluorescence of the different subpopulations were compared to the mean channels of the residual lymphocyte populations. Five surface markers were considered in performing this analysis: CD34, CD33, CD13, CD14, HLA-DR. A double morphological and morpho-immunological classification was performed by considering similar patterns of expression and by applying cluster analysis mathematical algorithms that are particularly effective in the definition of subgroups of observations with similar characteristics. We defined five different morpho-immunological subgroups with a concordance with FAB classification greater than 75%. Bright CD34 expression with weak to negative CD33 expression was more frequent among M0 and M1 AML while a bright CD34 and CD33 coexpression was found in a small subset of AML characterized by very poor prognosis: this group of leukemia may be the neoplastic counterpart of the small subset of normal CD34+ cells coexpressing CD33 which is characterized by a decrease capability to produce primitive colonies (LTIC and CFU-Bl). M2 and M4 AML had heterogeneous patterns of immunological properties: the former for the presence of CD34+ and/or CD34– blasts, the latter for the characterization of a low SSC myeloid population (CD34+ or CD34–, always CD33+) and a monocytic population (high SSC and brighter CD33 expression). On the basis of these data we can speculate that the analysis of both blast light scattering properties and morpho-immunological characteristics of intensity of expression along with a statistical analysis that defines subgroups of observation with similar characteristics, may help in the definition of further biological entities within FAB subtypes. In vitro and *in vivo* models of hemopoiesis may confirm the existence of these new proposed subgroups.

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FURTHER EVIDENCE OF MULTISTEP PATHOGENESIS AND CLONAL REMISSION IN A CASE OF MDS IN BLAST CRISIS

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The identification of AML patients with persisting clonal hematopoiesis in complete clinical remission has been considered as evidence of multistep pathogenesis of AML. According to the Knudson's model of *two hit* origin of malignancy, a first mutation gives origin to a phenotypically and karyotypically normal premalignant clone with a proliferative advantage; a later second *hit* gives rise to the malignant phenotype. Although recent reports conclude that clonal remission is rare in AML, the documentation of this entity remains one of the most convincing evidence for the multistep pathogenesis of hematological malignancies. We have analyzed the clonal status of a AML female patient during different phases of her disease, including the MDS phase, the blastic phase and the subsequent hematological remission achieved after intensive chemotherapy. To this purpose we have used the recombinant strategy based on the different methylation pattern, which allows to differentiate the active from the inactive X chromosome in subjects heterozygous for the DXS255 locus, recognized by the M27 probe. A diagnosis of RAEB-t was made in December 1992 in a 62-year-old female according to the FAB classification. At that moment cytogenetic studies were performed and disclosed the presence of 3 different clones: 46, XX; 46, XX, +double minutes (dm); 47, XX, +4, +d.m. Methylation analysis with M27 probe showed the presence of a single digested (unmethylated fragment), suggesting the clonal origin of bone marrow cells, whereas buccal mucosa cells exhibited a polyclonal methylation pattern. After 9 months overt AML (FAB M2) developed, with marrow myeloblasts accounting for 95% of nucleated cells. Karyotypic analysis showed 15 metaphases 47, XX, +4, + d.m., and 5 normal metaphases, whereas the X methylation pattern was unmodified. The patient went on to receive induction chemotherapy with ARA-C and Fludarabine after which she achieved complete hematological remission, with the restoration of a normal karyotype, confirmed by FISH studies. Surprisingly clonal analysis showed the persistence of clonal hematopoiesis on bone marrow and peripheral blood cell populations. The patient is in complete clinical and cytogenetic remission since October 1993, with constant clonal hematopoiesis. We can argue from these results that a sequence of multiple and superimposed genetic lesions occured in a hematopoietic progenitor leading to a clonal evolution. A first unknown hit caused the proliferation of a premalignant clone, which gave origin to a subclone gaining a proliferative advantage with the acquisition of d.m. and later of an extra 4 chromosome, probably responsible of clinical overt AML. Chemotherapy obtained the extinction of these latter subclones, with the reemergence of the premalignant clone, responsible of sustaining an apparent morphologically and cytogenetically normal hematopoiesis.

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BIOLOGICAL EFFECTS OF HEMOPOIETIC GROWTH FACTORS

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Hemopoietic growth factors (HGF) are cytokines acting through specific receptors at different levels of hemopoietic cell differentiation. Their actions can be summarized as follows:

- automaintenance of staminal progenitors;
- induction of proliferation and differentiation of progenitor and precursor cells;
- survival of hemopoietic (cells delaying cell death by apoptosis);
- activation of specific function of mature cells circulating in the blood or localized in the tissues.

Three facts are evident when the interaction of HGF with target cells is analyzed:

- each hemopoietic cell has receptors for several HGF;
- each HGF can act on different target cells;
- the interaction of HGF can have additive, synergic and antagonistic effects.

Therefore an ordered regulation of myelopoiesis requires the cooperation of several molecules.
Understanding the complexity of the system is crucial for a correct clinical use of single molecules or of their combination.
On these premises, the *in vivo* action of HGF on target cells will be discussed.

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GROWTH FACTOR RECEPTORS

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The functional pleiotropy and redundancy of growth factors can be explained on the basis of structural and functional characteristics of their receptors. Most growth factor receptors (GFRs) are multi-chain complexes with at least one specific and one "public" chain. This latter is usually responsible for signal transduction occurring following cytokine binding. The binding of a cytokine to its receptor implies the interaction of the cytoplasmic tails of the receptor chains to initiate a signalling cascade, through tyrosine kinase, and multiple signalling pathway activation, leading in most cases to activated forms of transcription factors (such as NF-IL-6, NF-kB) which can recognise DNA sequences on numerous regulatory genes. GFRs can be categorized into four types on the basis of structural homologies. Type 1: the so-called *hematopoietic cytokine receptor family*, IL-2R, IL-3R, IL-4R, IL-5R, IL-6R, IL-7R, IL-11R, IL-13R, GM-CSFR, G-CSFR, EpoR, LIFR; Type 2: IFNRs; Type 3: TNFR, FAS, CD27, CD30, CD40, IL-10R, NGFR. Type 4: IL-1R (belonging to the immunoglobulin superfamily).
The cytoplasmic tail of the receptor chains responsible for signal transduction in these GFR types are spatially associated with tyrosine-kinase molecules, as opposed to other GFR families (including EGFR, InsulinR, SCFR etc.), which have tyrosine-kinase motifs in their structure. Type 1 receptors are the most relevant to hematopoietic system. They can be classified into three sub-groups depending on the transduction-involved *public* chain they share, i.e. gp130 (for IL-6R, IL-11R, LIFR), common β chain (for IL-3R, IL-5R, GM-CSFR), common γ chain (for IL-2R, IL-4R, IL-7R, IL-9R, IL-13R). Type 3 receptors also represent an interesting family, since its members are implicated in complex functional features of lympho-hematopoietic cells. For example, CD30 is the specific marker of Th2-type CD4 cells and interactions with the CD30 ligand are likely to play a relevant role in Th2 switch of CD4 cells.
A large number of GFR chains are released from the cells and in most cases retain the capability of binding their ligands, thus providing an additional regulatory mechanism of cytokine activity.

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GROWTH FACTORS AND LEUKEMOGENESIS

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Growth factors (also named interleukins or cytokines) are soluble factors that mediate communication between cells in the hematopoietic system. They transmitt signals by interacting with specific receptors expressed on the surface of target cells. Most growth factors and their receptors have been identified at molecular level. Studies with recombinant molecules have revealed that a characteristic feature of growth factors is their functional pleiotropy and redundancy.
During the last few years the mechanisms involved in the cytoplasmatic transduction of growth factor signals have been largely elucidated. Growth factor receptors are associated with tyrosine kinases and growth factor stimulation of target cells is associated with tyrosine phosphorylation of cytoplasmic signalling proteins. Two major pathways of growth factor signalling have been identified: the *Shc/Grb2* signalling pathway, that is involved in RAS activation; and the JAK signalling pathway.
In the case of GM-CSF, ligand activation of target cells induces 1) rapid and reversible tyrosine-phosphorylation of *shc* proteins; 2) formation of stable *Shc/Grb2* complexes; 3) complexing of phosphorylated *shc* proteins with two novel phosphotyrosine-containing polypeptides of 140 kDa (p140) and 120 kDa (p120) approximately. The p120 is the product of the GM-CSFr β chain. GM-CSF stimulation induces tyrosine phopshorylation of the p120GMR and reversible association to the *Shc* proteins. The p140 is constitutively phosphorylated on tyrosine and associated to the grb2 protein. In the same cells the *grb2* protein form a stable complex with *sos*. Overexpression of Shc proteins in myeloid cells cells results in increased *Shc/Grb2*/p140-SOS complex formation and increased biological response to GM-CSF stimulation. These results suggest that recruitment of the Grb2/p140-SOS complex by phosphorylated *Shc* proteins is a critical event during GM-CSF signalling.
Shc proteins are constitutively phosphorylated and associated with the Grb2/p140/SOS complex in the fresh blasts of 80% of acute myelogenous leukemia cases.
These data suggest that the Shc/Grb2/p140-SOS signalling pathway is activated in a subset of human leukemias and that molecules that are involved in the regulation of Ras activity (receptors, cytoplasmic tyrosine kinases, phosphatases) are genetically altered in human leukemias.

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EFFECTS OF IL-6, IL-7 AND IL-12 ON LYMPHO-HEMOPOIETIC CELLS

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The main role of interleukin 6 (IL-6), interleukin 7 (IL-7) and interleukin 12 (IL-12) is to regulate differentiation and proliferation of lymphoid cells. Indeed, all three cytokines exert numerous and various activities on different cell populations. In particular, IL-6 has a pivotal role as an immune modulator of acute phase reaction; several other functions have been attributed to IL-6, including stimulation of B cell differentiation and antibody secretion as well as T-cell growth stimulation. IL-7 has been originally described as a proliferation factor for early-stage B lymphocytes. Further on, a stimulatory activity of IL-7 on T-cells has been reported. In addition, both IL-6 and IL-7, either alone or in association, may enhance the differentiation of cytotoxic T-cells. IL-12 is mainly involved in NK cell cytotoxicity upregulation. In particular, IL-12 is known to stimulate NK cell and LAK cell generation, as well as to enhance NK activity; in addition, an activating role on both T-helper cells and macrophages has been attributed to IL-12. Hence, IL-6, IL-7 and IL-12 are extensively involved in the regulation of the immune system. However, they should be considered as true multifunctional proteins since their biological activity is not restricted to the lymphoid lineages. Indeed, all three cytokines have been reported to be growth-stimulatory factors or, at least, *viability-factors* for early hemopoietic cells.
In order to further evaluate these functions, we took advantage of a simple method for the selection and identification of early pre-CFU-GM progenitors, based on the use of an immunotoxin (IT-CD71) originated by coupling an anti-CD71 MoAb to the SO6 toxin. Only non-cycling early progenitors survive following *in vitro* exposure to IT-CD71; however, the spared cells may grow in culture and generate committed progenitors. The activity of hemopoietic growth factors on *in vitro* survival and differentiation of immature IT-CD71 resistant progenitors was evaluated. A stimulatory role was documented for several early-acting cytokines, including interleukins primarily involved in the immune system.

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CORD BLOOD PROGENITORS AND RESPONSE TO GROWTH FACTORS

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Human cord blood (CB) is an attractive alternative to bone marrow as a source of hemopoietic progenitors cells since the number of CB progenitors is similar or even greater than that of normal bone marrow derived progenitors. Because gradient separation techniques, currently used to separate bone marrow, have produced poor results, in terms of progenitor cell recovery, when applied to CB separation, we have utilized a sequential separation over Emagel 33% (v/v) and Ficoll/Hypaque (n=21). In a smaller number of cases (n=6) CB mononuclear cells were loaded sequentially into AISMicroCELLector devices coated with soybean agglutinin (step 1) and with anti-CD34 (step 2). CB progenitors were tested for: 1) immunophenotype (CD34/DR, CD34/CD33, CD34/CD38); 2) expression of SCF, GM-CSF and IL-3 receptors; 3) mafosfamide resistance; 4) behaviour in short-term culture; 5) expansion in liquid culture in response to different CSFs combinations.
The percentage of cells CD34+/DR-, CD34+/CD33-, CD34+/CD38- was 1.2±0.2, 0.7±0.3 and 0.23±0.1, respectively. The receptor for SCF, GM-CSF and IL-3 was expressed on 89±7%, 42±11%, 35±9%of CD34-positive cells, respectively. CB cells showed a superior resistance to mafosfamide in comparison to BM cells. The mean values (range) of mafosfamide concentration inhibiting 50% of CB colony formation (ID50) were 73 μg/mL (65-83), 80 μg/mL (56-110) and 114 μg/mL (112-118) for CFU-GEMM, BFU-E and CFU-GM, respectively. Instead, the same figures in BM mononuclear cells were 49 μg/mL (23-79), 54 μg/mL (31-81), 60 μg/mL (36-81). The addition of SCF (50ng/mL) to culture medium resulted in a significant increase ($p \leq 0.05$) in mafosfamide ID50. Short-term cultures in methylcellulose (5×10^4 cells/mL) stimulated with IL-3 (10 ng/mL), G-CSF (10 ng/mL), GM-CSF (10 ng/mL), Epo (1U/mL) gave rise to 112±18 CFU-GM, 42±7 BFU-E and 14±4 CFU-GEMM; the addition of SCF (50ng/mL) resulted in a significant increase in CFU-GM (178±19, $p \leq 0.025$), BFU-E (53±7, $p \leq 0.375$) and CFU-GEMM (49±5, $p \leq 0.0005$) growth. CFU-GM expansion after 14 days liquid culture with the combinations IL3+GM-CSF+G-CSF+Epo and SCF+IL3+IL6 was 4.5 and 10.6 fold, respectively. Moreover IL-3 and IL-6 showed a synergistic effect in the presence of SCF.
In conclusion we can state that: 1) CB is a source of progenitor cells whose early ontogenetic level is demonstrated by growth factor receptor distribution, immunophenotype and mafosfamide resistance; 2) the feasibility of *ex vivo* amplification of CB progenitors might have implications for transplantation of adult patients as well as in the gene therapy setting.

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HEMATOPOIETIC GROWTH FACTORS (HGFs): CLINICAL APPLICATIONS AND FUTURE PROSPECTS IN HEMATOLOGIC MALIGNANCIES

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The past few years have seen an explosion in the identification, cloning and biologic characterization of HGFs and their receptors. The expanding knowledge of regulatory molecules with potent effects on hematopoiesis has been critical to the advancement of basic investigations into blood production, as well as to the development of new therapeutic approaches in the clinic. Three recombinant HGFs (EPO, GM-CSF, G-CSF) are today commercially available, and several others are currently undergoing clinical investigation (IL-1, IL-3, IL-4, IL-6, IL-11, PIXY, SCF). Incorporation of these molecules into the modern anti-tumor treatment strategies may provide important therapeutic benefits by exploiting their major potential effects. Thus, HGFs (GM-CSF, G-CSF) following chemotherapy or bone marrow transplantation have become integral part of many current therapeutic protocols for non-myeloid malignancies. The main benefit appears to be an accelerated recovery of granulocytes resulting in a reduction of the treatment-related morbidity (fewer days with fever, parenteral antibiotics; shorter hospitalization). Trials aiming at establishing the feasibility of dose intensification of chemotherapy by HGFs support are underway.
The possibility of enhancing the cytotoxic effects of chemotherapy by recruitment of quiescent leukemic cells cycle or by modulation of intracellular drug metabolism appears realistic, as suggested by early data on HGFs priming in AML. New methods to support myeloablative treatment strategies are offered by the autologous transplantation of peripheral blood hematopoietic precursor cells mobilized by HGFs.
Other applications of potential benefit include: induction of terminal differentiation of leukemic blasts in AML and myelodysplasia by HGFs, given alone or in combination with other differentiating agents; inhibition of growth factor-dependent proliferation of neoplastic plasma cells in myeloma by anti-IL-6 monoclonal antibody. Defining the future of HGFs in the clinic will require exploration of two therapeutic paths. Given the limitations and expense of cytokine treatment it is crucial to identify their most efficient use; alone or in combination with newer molecules (SCF, IL-6, IL-11) or cellular therapies (peripheral blood progenitor cells). The monetary costs should also stimulate further research into how to use these growth factors most effectively with the aim of targeting therapy to those patients who need them the most.

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IN VITRO HIV-1 INFECTION OF HEMATOPOIETIC PROGENITORS IN UNICELLULAR CULTURE

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Uni- or multi-lineage suppression of hematopoiesis is observed in the majority of AIDS patients. The mechanism(s) underlying these abnormalities is not understood: particularly, the HIV infection of hematopoietic progenitors and stem cells (HPCs/HSCs) is highly controversial. We report that individual CD34+ HPCs from adult peripheral blood (PB) are susceptible to *in vitro* HIV infection.
Primitive CD34+ HPCs, stringently purified from PB and grown in liquid phase multilineage differentiation culture supplemented with saturating amounts of c-kit ligand, IL-3, GM-CSF and erythropoietin, were HIV-1 infected at different days (from day 1 through 5) and then cloned in single cell methylcellulose culture. Incubation with either HTLVIIIB or 8 E.5 cell line supernatant at a multiplicities of infection (MOI) ranging from 0.01 to 10 TC1D50/cell did not modify the number of colonies generated by HPCs, i.e., erythroid burst-forming units (BFU-E), granulocyte-macrophage colony-forming units (CFU-GM) and multilineage CFUs (CFU-GEMM). The colonies were analyzed for the presence of HIV proviral DNA or tat-specific mRNA by PCR or reverse transcription PCR respectively and HIV proteins by ELISA. Initially viral DNA sequences were detected in 5-10% of CFU-GM colonies. In further experiments tat mRNA transcript was found in ~20% of both BFU-E and CFU-GM colonies; no evidence of HIV infection was present in CFU-GEMM colonies. A minority of CFU-GM and BFU-E colonies were positive for the p24 viral protein.
Purified HPCs, grown in liquid phase unilineage monocytic differentiation culture, were susceptible to HIV infection and mRNA expression throughout the differentiation/maturation process.
Double labeling of CD34/CD4 membrane antigens was demonstrated on 10-15% of the purified cells from day 0 through 5, thus suggesting their potential susceptibility to HIV-1 infection.
These observations strongly indicate the HIV infection susceptibility of a minority of primitive HPCs, possibly through the CD4 membrane antigen. Thus, they reflect on the hematopoietic abnormalities in AIDS patients, while providing an experimental model to test gene therapy approaches against hematopoietic HIV infection.

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STROMAL CELLS FROM LYMPHOID TISSUE CAN BE INFECTED BY HIV 1

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The pathological changes occuring in the lymph nodes (LN) of HIV 1 infected patients ultimately result in the loss of both LN structure and function. Studies demonstrating HIV 1 infection of lymphocytes, macrophages and follicular dendritic cells in the LN suggest that additional LN-derived cell types may also contribute to HIV 1 mediated pathology. In particular the LN stromal elements bearing fibroblastic characteristics are of mesenchymal origin and are considered critical to the LN structure through their production of, and association with, bundles of connective fibers and represent one such potential target of HIV 1 infection.
Accordingly, we have investigated the susceptibility of LN-derived stromal cell lines (HTSC) to HIV infection using a combination of electron microscopy, HIV 1 specific mAb, and PCR analyses to detect HIV 1 associated DNA sequences.
Our results demonstrate that HTSC are efficiently infected following co-cultivation with the HIV 1 infected lymphoblastoid cell line GY1. Infected stromal cells contain intracellular viral particles present as free virus or associated with phagocytic vesicles. These particles express the HIV 1-specific p24 antigen as assessed by immunohistologic analyses using a monoclonal, HIV-specific, anti-p24 antibody. Moreover, PCR analysis of genomic DNA isolated from particle-bearing tonsillar stromal cells identified HIV 1-specific sequences not present in either uninfected stromal cells or uninfected GY1 cells. Furthermore these HIV 1 genome bearing HTSC can transmit infection to the HIV 1 sensitive T cell line A301 added to the cultures. The mechanism by which HIV 1 infects HTSC remains unclear, but, does not appear to be CD4-mediated. None of the human tonsillar stromal cell lines express this cell surface antigen as assessed by flow cytometry, immunohistochemistry and RT-PCR with nested primers analyses.
Taken together, these results demonstrate that HIV 1 infected lymphocytes can transfer viral particles to tonsillar stromal cells, and that subsequent to transfer, the viral genome is reverse transcribed, and integrated into the stromal cell DNA. The resulting infected HTSC can transmit infection to HIV 1 sensitive cells. The biological consequences of HTSC infection remain unclear, however, these cells may contribute to HIV 1-mediated pathogenesis indirectly as a viral reservoir or directly by structural and functional modification of the lymphoid microenvironment.

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MAPPING OF CYTOKINE EXPRESSION IN AIDS-RELATED NON HODGKIN LYMPHOMA (AIDS-NHL)

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AIDS-NHL represent one of the most common AIDS-related malignancies, are derived from B cells and are classified into two main histotypes, small non cleaved cell lymphoma (SNCCL) and diffuse large cell lymphoma (DLCL). AIDS-lymphomagenesis is thought to procede through two phases. In the first phase, host factors pre-existing to lymphoma development, including deregulated cytokine production, antigen stimulation and infection by Epstein-Barr virus, would cause the polyclonal proliferation of genetically normal B cells. In a second phase, multiple genetic lesions would accumulate within a single clone thus leading to AIDS-NHL. The role of cytokines in AIDS-NHL development is relatively unexplored. In order to unequivocally define it, pure AIDS-NHL populations are required. Toward this aim, we have applied the RT-PCR technique to map cytokine expression (IL-1α, IL-1β, IL-2, IL-4, IL-6, IL-8, hIL-10, TNFα, TNFβ, TGFβ2 and IFNγ) in a panel of AIDS-NHL cell lines representative of both AIDS-SNCCL (n=5) and AIDS-DLCL (n=2). Cell lines of NHL of similar histology but derived from the immunocompetent host were also tested (3 sporadic SNCCL and 3 endemic SNCCL). We aimed at defining: 1) whether distinct patterns of cytokine expression correlate with different AIDS-NHL histologies; and 2) whether the pattern of cytokine expression varies between AIDS-NHL and NHL of similar histology derived from the immunocompetent host. Our data show that expression of IL-10, IFNγ and IL-6 is a common feature of both AIDS-SNCCL and AIDS-DLCL, whereas expression of IL-1α, IL-1β, IL-8 and TGFβ_2 is consistently negative in both histotypes. The expression of TNFα, TNFβ, IL-2 and IL-4 seems to depend upon the AIDS-NHL histologic type. TNFα, TNFβ and IL-4 are expressed by AIDS-SNCCL but not by AIDS-DLCL. On the contrary, IL-2 expression is restricted to AIDS-DLCL. When AIDS-SNCCL is compared to SNCCL of the immunocompetent host, it is of note that IL-6 and IL-4 expression appears to be restricted to AIDS-SNCCL, whereas TNFα, TNFβ and IFNγ are widely expressed by both AIDS-SNCCL and SNCCL of the immunocompetent host. Finally, hIL-10 expression is shared by AIDS-SNCCL and sporadic SNCCL, but not by the endemic SNCCL tested.
The different pattern of cytokine expression observed in AIDS-SNCCL and AIDS-DLCL further substantiate the notion that the pathogenesis of these two AIDS-NHL types follows distinct molecular and cellular pathways. In addition, the molecular and immunophenotypic resemblance of AIDS-SNCCL with sporadic - and not endemic - SNCCL of the immunocompetent host is also confirmed by the distribution of hIL-10 positivity. Expression mapping of newly identified cytokines (IL-13, IL-14, IL-15) is currently in progress.

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QUANTITATION OF VIRAL NUCLEIC ACIDS IN HIV-1-INFECTED INDIVIDUALS BY COMPETITIVE PCR TECHNIQUES AS A TOOL FOR MONITORING DISEASE PROGRESSION AND EFFICACY OF ANTIVIRAL THERAPY

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Several experimental and clinical evidences indicate that, during the course of HIV-1 infection, progression from asymptomatic state to AIDS is concomitant with an increase in the number of infected CD4 cells, in the level of viremia, in the accumulation of virus in lymphoid tissues, and in the expression of the integrated provirus. Therefore, it is important to monitor the amount of viral DNA and RNAs in infected individuals.
We have developed a technique for the absolute quantitation of viral nucleic acids in biological samples, based on competitive PCR (for DNA) and RT-PCR (for RNA) procedures (1-4). This technique allows to exactly determine the number of molecules of provirus (as compared to molecules of a single copy cellular gene), of viral RNA in serum (viremia), and of viral transcripts in infected cells (as compared to molecules of a constitutively expressed cellular mRNA). As far as the latter are concerned, the method also allows the recognition of different classes of processed RNAs, produced by differential splicing of the genome-length viral messenger.
The competitive PCR procedure entails the addition to the sample of competitor DNA or RNA molecules, having the same primer recognition sequences as the target viral nucleic acids but differing in size. Since the amount of competitor is known, the amount of viral target can be easily derived from the ratio between of the two amplification products (competitor/target). The method is immune to any variable affecting conventional PCR, is independent of amplification cycle number, and is not influenced by overall efficiency of the reaction.
By this method, we are currently monitoring several samples derived from serum, PBMCs and lymphoid tissues of infected individuals, in order to correlate disease progression with viral load and efficacy of conventional and non conventional antiretroviral therapies.

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β-INTERFERON COMBINED WITH AZIDOTHYMIDINE IS LESS SUPPRESSIVE ON NORMAL BONE MARROW PROGENITORS THAN α-INTERFERON COMBINED WITH AZT

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The combination of IFNα and azidothymidine (AZT) has been used in HIV positive patients to potentiate the antiretroviral effect of AZT. However a significant myelotoxicity has frequently been observed as a severe clinical complication. A synergistic suppressive effect on normal hemopoietic progenitors has also been shown *in vitro* at concentrations comparable to the plasma levels attained in patients treated with IFNα/AZT combined therapy.
Since IFNβ appears to be both *in vitro* and *in vivo* less myelotoxic than IFNα, the present *in vitro* study was performed to compare the suppressive effects of IFNβ and IFNα, both alone and in combination with AZT, on normal committed granulopoietic (CFU-GM), erythropoietic (CFU-E/BFU-E) and on multipotential progenitors (CFU-GEMM) from 10 bone marrow donors.
A dose-dependent inhibition was demonstrated both for IFNα and IFNβ with a higher sensitivity of erythropoietic and mixed progenitors. However, comparing the IFNs, at the intermediate concentration employed (500 U/mL), IFNβ was significantly less suppressive than IFNα on CFU-GM (p=0.005), BFU-E (p=0.0008) and CFU-GEMM (p=0.0497) progenitors.
When the IFNs were combined with AZT, their inhibitory effects were strongly enhanced, however the lower suppressive activity of IFNβ with respect to IFNα was confirmed even when they were tested in association with AZT.
These data suggest that the treatment with IFNβ plus AZT might be a useful alternative to IFNα/AZT in the management of HIV positive patients in order to provide an effective antiviral protection and reduce the myelotoxicity.

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ABNORMALITIES IN THE EXPRESSION OF SURFACE MARKERS IN CIRCULATING CELLS FROM HIV-INFECTED PATIENTS

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Peripheral blood cells derived from 15 HIV-infected patients (10 asymptomatic infection, 3 ARC, 2 AIDS) and 5 control subjects were analyzed by flow cytometry using a large panel of monoclonal antibodies (mAbs) recognising relevant surface molecules associated with lymphocytic (CD4, CD8, CD19, CD3/HLA-DR, CD8/CD45, CD8/HLA-DR, CD8/CD38, CD54-ICAM1), monocytic (CD14, CD45, HLA-DR, CD11b-CR3, CD11c, CD13, CD35- CR1), and neutrophil differentiation (CD45, CD11b, CD11c, CD13, CD35, CD54). The mean absolute number of CD4+ cells was, respectively, 440/mmc and 100/mmc in asymptomatic and ARC-AIDS patients.
The results showed a significant reduction in either the percentage or mean intensity of fluorescence (MFI) of monocyte positivity for CD14 and HLA-DR, while neutrophil granulocytes showed a significant increase in the MFI of CD11b, CD35 and CD54. As far as the lymphocyte' surface marker profile is concerned, we observed an increase in the percentage of positive cells for the following combinations of mAbs: CD3+/DR+, CD8+/CD45+, CD8+/DR+ and CD8+/38+.
In conclusion, this study indicates that HIV-infected patients with advancing stage of the disease show multiple abnormalities in the expression of various immunophenotypic markers, which would result in a severe disturbance of the immunoregulatory network typical of patients with full-blown AIDS. The possibility of using these molecules as markers of progression of the disease is also postulated by the Authors.

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GROWTH AND DISSEMINATION OF THE HUMAN PROMYELOCYTIC LEUKEMIA NB4 CELL LINE IN SCID MICE

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A human acute promyelocytic cell line (NB4) bearing the specific translocation t(15;17) has been grown in the severe combined immune deficiency (SCID) mouse. Three-four weeks after subcutaneous injection of 10×10^6 NB4 cells, a palpable tumor grew only at the site of injection in all injected mice. In contrast, after intravenous injection, macroscopically disseminated tumor developed. This was characterized by large paraspinal masses and frequent massive involvement of ovaries. At microscopic examination, leukemic infiltrates were consistently observed in the lung, bone marrow, liver and meninges. Kidney and spleen were usually not involved. There was no difference in the tumor take rate and rapidity of growth between NB4 and NB4 ATRA-resistant cell lines in SCID mouse. NB4 cells derived from excised tumors did not differ in terms of phenotype and chromosomal abnormalities (modal number and structural rearrangements) from the original NB4 cell line, indicating that tumor growth in the SCID model did not induce additional genetic events or clonal selection. Our NB4/SCID model does not exactly mimick the clinico-pathological features of APL in human (e.g. DIC syndrome and signs of myeloid maturation are absent), probably due to the fact that the NB4 cells are highly indifferentiated and can mature only in the presence of RA. In spite of this, we are now using this model for the following purposes:
a) optimize RA treatment strategies including schedules and dosing;
b) test the ability of the RA and new retinoids to restore maturation *in vivo* both under condition of high tumor burden and minimal residual disease;
c) study the mechanism of resistance to treatment with RA; d) Explore new therapeutic modalities based upon the use of RA plus chemotherapy or antisense oligonucleotides.
Finally, our SCID model shows that PCR identification of PML/RAR transcripts and the immunohistochemical analysis of mouse tissues with monoclonal antibodies directed against the human CD68 molecule (KP1) and PML gene product (PG-M3) provide an excellent tool for quantifying minimal residual disease and monitoring the response to the above therapeutic procedures.

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PG-M3: A NEW MONOCLONAL DIRECT AGAINST THE ACUTE PROMYELOCYTIC LEUKEMIA (PML) GENE PRODUCT

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PG-M3 is a new monoclonal antibody (mAb) specifically directed against a peptide sequence (PSPSPTERAPASEEC-NH2) located close to the highly conserved, cysteine/histidine rich region (the putative DNA-binding domain) of the PML protein, the product of the normal PML (promyelocyte) gene that fuses with the gene encoding the retinoic acid receptor (RAR) in the t(15;17) chromosomal translocation of acute promyelocytic leukemia (APL). The epitope recognized by PG-M3 is shared by most PML protein variants including wild-type PML isoforms, aberrant PML and PML/RAR fusion proteins, it is fixative sensitive, species-specific and, as expected, located within the nucleus. The nuclear staining pattern of PG-M3 varies from "speckled" (cells other than APL) to "micropunctate" (APL cells). The last staining pattern is reverted to "speckled" following retinoic acid treatment. Cells transfected with cDNAs encoding physiologically deleted forms of PML show complete or partial restriction of the PG-M3 staining to cytoplasm. This finding provides the first immunocytochemical evidence of the putative nuclear localization signal (NLS) of PML. PG-M3 produces a "speckled" nuclear staining of a wide variety of cells in normal human tissues. Within the lymphohemopoietic system, absent or low expression of PML is observed in immature cortical thymocytes and germinal center B-cells; the significance of this finding is unknown. Cells showing the highest expression of PML are epithelial cells, endothelial cells and tissue macrophages (especially activated ones). In keeping with this finding, we found increased expression of PML in the U937 promonocytic cell line, following exposure to agents inducing activation (IFN-) and/or maturation (vitamin D3 plus TGF). This finding warrants further studies on the role of PML in the process of monocyte/macrophage activation and maturation. In conclusion, the PG-M3 mAb represents a valuable tool for research and diagnosis of APL.

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THE RETINOID DERIVATIVE 4-HPR INDUCES APOPTOSIS AND BY-PASSES RESISTANCE TO ALL-TRANS RETINOIC ACID (ATRA) IN HEMOPOIETIC CELLS

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N-(4-hydroxyphenyl)retinamide (HPR) is a synthetic retinoid with cancer chemopreventive activity. We have examined the "*in vitro*" effects of HPR on lymphoid and myeloid malignant cell lines and found that between 10 uM and 0.3 uM it induces dose-dependent growth inhibition. Such strong effect was not observed with equimolar doses of ATRA. Time-course analysis showed that 3 uM or less HPR induces a rapid decrease of thymidine uptake and viability (> 90%), whereas doses between 1 and 0.3 uM produce a cytostatic effect. Interestingly, the RA-resistent HL60-R and NB306 cells, characterized by a defect in the RA receptor and lack of pml/rar protein, respectively, were, like the parental RA inducible HL60 and NB4 cell lines, fully responsive to HPR.
The apoptotic effects of HPR were evidenced by flow cytometric analysis of cell cycle and ultrastructural examination of HPR-treated samples. Moreover, DNA fragmentation characteristic of apoptosis was visualized by gel electrophoresis as well as by "in cell" enzymatic labelling with fluorescent δUTP of DNA breaks.
In conclusion, this study demonstrates that HPR strongly suppresses malignant cell growth and triggers apoptosis by a mechanism apparently different from that induced by retinoic acid. We are currently investigating the role of the apoptosis-associated genes bcl-2, bax and MCL-1 in HPR-induced cell death.

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ASSESSMENT OF MOLECULAR STATUS IN LONG SURVIVAL ACUTE PROMYELOCYTIC LEUKEMIA PATIENTS BY REVERSE TRANSCRIPTION POLYMERASE CHAIN REACTION OF PML-RAR FUSION mRNA

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The characteristic t(15;17) translocation of acute promyelocytic leukemia (APL) fuses genes encoding PML on chromosome 15 and the nuclear retinoic acid receptor (-RAR) on chromosome 17.
The fused mRNA PML-RAR can be detected by a newly described reverse transcribed-Polymerase Chain Reaction (RT-PCR). Using sequence specific primers induced RT-PCR, we serially evaluate bone marrow aspirate from 10 patient with APL, characterized by a long disease-free status after chemotherapy induction and consolidation (median 61.5 months; range from 33 months to 101). All the patients were in clinical and cytogenetic remission at the time of molecular evaluation (range from 31 to 89 months from CR). All but one patient were found RT-PCR negative at the molecular level for the expression of PML-RAR transcript suggesting that long- term survival of APL is associated with the eradication of the neoplastic clone. The only one female who was PML-RAR positive until 32 months after achievement of CR, was persistently PCR positive in different time collected samples analysed (+13 months, + 15 months, +32 months).
These observations suggest that in rare case a complete remission may be maintained despite the presence of expression of the PML-RAR transcript. A quantitatively assessment of the amount of neoplastic transcript in PML patients may be helpful to detect the risk of clinical relapse in patients with minimal residual disease.

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ATRA+ANTRACYCLINES IN PATIENTS WITH ACUTE PROMYELOCYTIC LEUKEMIA: HEMOSTATIC ASPECTS

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Sixteen patients, 9 males and 7 females, mean age 39 years (range 14-75) affected by acute promyelocytic leukemia (APL) at diagnosis (n.13) or in relapse (n.3) have been treated with ATRA (45 mg/m^2/day). Six patients received, in addition, one to three administrations of Daunorubicin or Idarubicin. Clinical and hemostatic laboratory parameters have been monitored during the first month of treatment. Hemorrhage was present at the beginning in 6 patients (WHO grade 1 or 2). During treatment another patient had hemorrhage (WHO 1), while 1 case of pulmonary embolism has been registered. A patient with acute hepatic failure showed on day +4 a clinical pattern of VOD and he dead on day +9. Four patients developed the ATRA-syndrome. Eleven patients received platelets and/or plasma support. The mean value of aPTT was always in the normal range, while PT was significantly reduced until day +10. The plasma fibrinogen, always more than 100 mg/dL, was normal in the majority of the patients. The basal levels of plasma fibrin monomers and of D-Dimer were elevated but gradually decreased to normal values on day +15. Between days +15 and +25 a further increase of the same parameters was observed in some patients. An interesting relationship between these two parameters of blood coagulation and fibrinolysis activation and the number of white blood cells has been found, suggesting a crucial role of white blood cells in the coagulopathy of APL patients receiving ATRA.

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IN PROMYELOCYTIC BLAST CRISIS OF CHRONIC GRANULOCYTIC LEUKEMIA DUAL COLOR FISH DETECTS BOTH THE T(9;22) AND THE T(15;17).

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A 62-year-old-man came to our observation on May '94. On physical examination a large splenomegaly was noted. Laboratory findings showed hyperleucocytosis with atypical promyelocytes in peripheral blood and bone marrow, and DIC.A two drugs regimen consisting of Ara-C 200 mg/12h for seven days and daunorubicine 80 mg/24h for three days was started at once with only a temporary decrease of leukocytes; therefore a new protocol including Ara-C at the same dosage and mitoxantrone 18 mg/24h for three days followed by ATRA 80 mg/24h was dispensed and a longer control of leukocytosis was achieved;the patient is still on treatment. A cytogenetic analysis performed at onset revealed that 70% of marrow cells carried both the t(9;22) and the t(15;17) translocations; therefore a diagnosis of promyelocytic blast crisis of CGL was made. Noteworthy 30% of the cells showed the Ph1 chromosome only. For this reason we decided to apply dual color FISH to evaluate if the t(15;17) translocation was confined to the sole granulocytic cell lineage. The t(9;22) as well as the t(15;17) translocations DNA probes are mixtures of digoxigenin labeled cosmid probes,specific for both the 22q11 and the 17q21 chromosome breakpoints,and of biotin labeled cosmid probes,specific for the 9q34 and for the 15q22 chromosome breakpoints. Both the probes were reported to flank the fusion in cases of CGL and APL respectively. To combine morphologic and FISH studies marrow slides were prefixed in methanol:acetic acid 3:1 for three times of ten minutes each.Hybridized probes were detected with rhodamine (red signal at 22q11 and at 17q21 locus) and with fluorescein (yellow-green signal at 9q34 and at 15q22 locus) and nuclear DNA was counterstained with DAPI (blue). A combined red/green spot was indicative of both der (22)t(9;22) and of der(17)t(15;17). Dual color FISH pointed out that only leukemic cells retained both the t(9;22) and the t(15;17),this last being absent in all the other marrow cells. Therefore FISH suggest that the t(15;17) is secondary to the Ph1 chromosome.

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INTERACTION OF STEM CELL FACTOR (SCF), INTERLEUKIN 3 (IL3) AND BASIC FIBROBLAST GROWTH FACTOR (bFGF) IN THE REGULATION OF NORMAL AND PRELEUKEMIC MEGAKARYOCYTOPOIESIS

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Megakaryocyte (Mk) progenitor cell proliferation and differentiation is regulated by a variety of interacting growth factors, including GM-CSF, IL3, IL6, IL11 and SCF. Basic fibroblast growth factor (bFGF), a multifunctional growth factor produced by cellular components of the bone marrow microenviroment, has been recently shown to promote the proliferation of normal human megakaryocyte progenitor cells (CFU-Mk and BFU-Mk).

We evaluated the effect of human recombinant bFGF (10 to 200 ng/mL) alone or in combination with other hemopoietic growth factors (IL3 10 ng/mL; IL6 40 ng/mL; IL11 10 ng/mL, GM-CSF 20 ng/mL) on the proliferation of CFU-Mk and BFU-Mk from bone marrow (BM) and peripheral blood (PB) of normal subjects and myelodysplastic (MD) patients. MD syndromes are a group of clonal disorders characterized by peripheral cytopenias and hypercellular marrow. The growth of CFU-Mk is poor, even when the pltelet number is only slightly decreased.

1×10^4 LD or 5×10^3 CD34 + BM or PB cells were grown in a standard plasma clot assay in the presence of IL3, IL6, IL11, GM-CSF, SCF and bFGF as single factor or in combination. CFU-MK were evaluated by immunofluorescence (with a monoclonal antibody recognizing the IIb/IIIaGP) after 12 days of incubation; BFU-Mk were evaluated after 21 days of culture. the mean number of megakaryocytes per colony were also counted.

The growth of normal Mk progenitors increased when SCF was added to IL3, IL6, IL11 or GM-CSF. Basic FGF, in association to IL3, GM-CSF, IL6 or IL11 did increase the number and the size of CFU-Mk and BFU-Mk; however, it did not seem to synergize with SCF.

In the majority of MDS patients CFU-MK were most detectable, even when the combination of all the factors was used. In 8/23 cases CFU-Mk were detectable in the presence of IL3. The addition of SCF enhanced CFU-Mk growth (130 to 256%). In 6/23 ceases CFU-Mk were detectable also in the presence of bFGF alone. In these cases bFGF was synergic with IL3.

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ALL-TRANS RETINOIC ACID (ATRA) POTENTIATES MEGAKARIOCYTE COLONY FORMATION: IN VITRO AND IN VIVO EFFECTS AFTER ADMINISTRATION TO ACUTE NON LYMPHOID LEUKEMIA PATIENTS

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We evaluated the *in vitro* growth of normal hematopoietic progenitors (CFU-GM, CFU-GEMM, CFU-meg) stimulated by optimal sources of colony stimulating activity in the absence or presence of 10^{-6}M all-trans retinoic acid (ATRA). ATRA alone did not show any colony stimulating ability (CSA), while it significantly increased the number of CFU-GM (p=0.003) and both the number (p=0.009) and the size (p=0.002) of CFU-meg in the presence of appropriate colony stimulating activity. In parallel experiments, the *in vitro* growth of the different hematopoietic progenitors was compared in 28 patients affected by acute non lymphoid leukemia (ANLL), mainly acute promyelocytic leukemia (APL). Cells were harvested after remission induction obtained:

a) with ATRA, followed by one chemotherapy cycle (CHT) (3/7: daunorubicin + Ara-C), administered to reinforce complete remission: Group A - 10 APL (ATRA/CHT).

b) One CHT cycle (3/7 as above): Group B - 8 APL (APL/CHT).

c) One CHT cycle (3/7 as above): Group C - 10 ANLL not APL (ANLL/CHT). The number of the different hematopoietic progenitors, and in particular CFU-GM and CFU-meg, was significantly higher in APL patients treated with ATRA plus CHT (Group A) with respect to APL (Group B) or ANLL non APL (Group C) patients treated with CHT alone (CFU-GM: p=0.01; CFU-meg: p=0.003).

Remarkably, the ability of ATRA to stimulate both *in vitro* and *in vivo* megakaryocytopoiesis matches with the described reduced duration of hematological recoveries post-CHT in patients induced to remission with ATRA. Thus, our data suggest that the *in vivo* administration of ATRA could be beneficial for a faster recovery of normal residual hematopoiesis and in particular megakaryocytopoiesis, frequently impaired in ANLL after intensive CHT, and especially after autologous bone marrow transplantation.

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PROLIFERATIVE POTENTIAL AND PHENOTYPE OF HEMOPOIETIC PROGENITORS COLLECTED BY LEUKAPHERESES FROM SEVERE APLASTIC ANEMIA (SAA) PATIENTS AFTER PROLONGED G-CSF TREATMENT

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Seventeen SAA patients were treated according to an ongoing EBMT trial designed for patients presenting with less than 0.5×10^9/L. This protocol includes horse ALG, 6-methylprednisolone, CyA and G-CSF (5 ug/kg/die) from day +6 to day +90.
Each patient underwent 1 to 8 leukapheresis (median 5) from day +30 to day +90 of G-CSF treatment. The results per procedure were: MNC 6.35×10^8/kg (range 0.8-18.7) CFU-GM 1.2×10^4/kg (range 0-39) CD34+ 1.6×10^6/kg (range 0.08-5.69 Twenty leukapheresis were tested in long term cultures and grew LTC-IC (32/kg, range 0-1050); three of them also contained HPP-CFC in variable amount. Samples from six leukapheresis were studied after FACS separation for CD34+ cells: the mean proportion of CD34+CD15–DR– cells was 0.067% and of CD34+Lyn– 0.173%. Only one sample contained CD34+Lyn–Thy1+ cells.
This study shows that committed and pluripotent hemopoietic progenitors can be mobilized in the peripheral blood of patients with acquired SAA after ALG+CyA+prednisolone and prolonged administration of G-CSF. Two fallouts of this observation seem possible: firstly, autologous hemopoietic recovery after ALG+G-CSF may be due to reseeding of hemopoietic progenitors, and secondly, these cells may be capable of allowing sustained hematopoiesis if infused after high dose chemotherapy.

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NORMAL AND LEUKEMIC CD34-POSITIVE PROGENITORS FROM CHRONIC MYELOGENOUS LEUKEMIA PATIENTS HAVE A DIFFERENTIAL CAPACITY TO ADHERE TO ALLOGENEIC STROMA

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Chronic myelogenous leukemia (CML) is a clonal disorder characterized by the co-existence of Ph-neg with Ph-pos progenitors. We have previously demonstrated that marrow-derived CML cells with maintained capability of adherence to allogeneic stroma are significantly enriched in Ph-neg progenitors (35±6 vs 15±4, P≤.005) and mafosfamide incubation is additive with stroma-adherence in enriching for Ph-neg progenitors (58±9% vs 35±6%, P≤.005). In order to confirm that stroma-adherent Ph-neg progenitors derive from a primitive population, experiments were performed with CD34-enriched cells, isolated through an immunoadsorption technique using tissue culture flasks (AIS, MicroCELLector™) coated with an anti-CD34 antibody. CD34+ cells (1×10^5/mL) were plated onto confluent stroma for 2 hours (37°C, 5% CO_2) and nonadherent cells were removed by extensive washing. Following a short-term (3 days) liquid culture, stroma-adherent cells were harvested, incorporated in methylcellulose and individual colonies were analyzed by single colony karyotyping and and fluorescent *in situ* hybridization (FISH) with a biotinylated cosmid DNA probe that hybridize to abl oncogene. CD34 enrichment resulted in a population that was on average 74% pure. The mean (±SEM) percentage of CD34+ cells able to adhere to allogeneic marrow stroma was 58±15%. Experiments (n= 5) evaluating the number of progenitor cells in the non-adherent fraction, showed that 31±6% of CML progenitors attached to stroma during the 2-hour adherence. The input numbers (mean ± SEM) of CFU-GM per 1×10^4 untreated and mafosfamide-treated cells plated onto marrow stroma were 1779±501 and 408±265, respectively. The mean (± SEM) output of progenitors generated by 10,000 CD34+, stroma-adherent cells was 888±188 and 570±258 for untreated and mafosphamide-treated cells, respectively. On direct cytogenetic analysis, the mean (±SEM) percentage of Ph-neg metaphases was 5±5%. CD34+ cells generated 22±10% Ph-neg clones. By combining CD34 selection and stroma-adherence, the percentage of Ph-neg clones could be increased up to 38±14%. The CD34+, stroma-adherent, mafosfamide-treated fraction generated 56±18% Ph-neg progenitors. Interestingly, the combination of mafosfamide treatment and CD34 selection with stroma-adherence did not result in a significant improvement of Ph-neg clones (46±18% vs 56±18%, P≤.375). FISH confirmed the results obtained by conventional cytogenetic analysis. Single colony transfer experiments revealed that 50±4% stroma-adherent and 70±4% stroma-adherent mafosfamide-treated progenitors gave rise to secondary colonies.
In conclusion, the present data demonstrate that Ph-neg clones with maintained stroma-adherence derive from the CD34+ fraction, are mafosfamide resistant and have high-replating potential.

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ANTIPROLIFERATIVE ACTION OF THE PROTEIN-TYROSINE KINASE INHIBITOR GENISTEIN ON NORMAL AND LEUKEMIC HEMATOPOIETIC PROGENITOR CELLS

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Protein-tyrosine kinases (PTKs) mediate critical aspects of cellular signalling associated with cellular proliferation and differentiation. It was the aim of the present study to evaluate the effect of the natural PTK inhibitor genistein (Sigma, Mo, USA) on hematopoietic progenitors obtained from consenting normal donors (n= 3) and chronic myelogenous leukemia (CML, n= 3) patients. To investigate the effect of genistein, three cell fractions were used: light-density mononuclear cells (MNCs); soybean agglutinin-negative (SBA-neg) cells; highly purified CD34++CD45RA– cells, obtained by fluorescent-activated cell sorting (FACSort, Becton-Dickinson). Multilineage (CFU-GEMM), erythroid (BFU-E) and granulopoietic (CFU-GM) progenitors were assayed in methylcellulose cultures by plating test cell suspensions at appropriate concentrations. Cultures were stimulated with IL-3, G-CSF, GM-CSF and Epo. Increasing doses (1-100 μM) of genistein induced a statistically significant (P ≤0.05), dose-dependant suppression of colony formation from normal CFU-GEMM, BFU-E, and CFU-GM generated by MNCs, SBA-neg cells and CD34++ CD45RA– cells. Inhibition curves compared by single-factor ANOVA were not statistically different. For normal progenitors, genistein concentrations inducing 50% inhibition (ID50) of colony formation ranged from 18 to 53 μM. Similarly, genistein (1-100 μM) suppressed in a dose-dependant manner colony formation from SBA-neg, CML-derived CFU-GEMM, BFU-E, and CFU-GM (ID50 values: 20, 28, and 40 μM, respectively). These values were not significantly different from those calculated for normal progenitors. Preincubation experiments revealed that a one-to-two-hour exposure of CML MNCs to genistein (100 μM) followed by repeated washings and methylcellulose culture induced a significant suppression of colony formation (range: 30 to 100%).
In conclusion, the present data demonstrate that:
(a) continuous exposure to genistein induces a marked inhibition of mature and primitive hematopoietic progenitors;
(b) this inhibitory effect is also evident when hematopoietic cells are transiently exposed to genistein, thus showing an irreversible effect of genistein on cell proliferation.
The therapeutic potential of PTK inhibitors will require further investigation.

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HIGH-DOSE CHEMOTHERAPY FOLLOWED BY ERYTHROPOIETIN PLUS G-CSF FOR STEM CELL MOBILIZATION

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Stem cell mobilization for autotransplantation is now feasible in many patients with the administration of high-dose chemotherapy followed by single or combination cytokines. Erythropoietin (EPO) is a cytokine which has shown an interesting activity also on non erythroid progenitors, but its potential in stem cell mobilization has not been, at present, sufficiently investigated. In our study we assessed the feasibility of priming with high-dose chemotherapy followed by the combination EPO plus G-CSF; we also evaluated the efficacy of EPO plus G-CSF in increasing peripheral hemopoietic progenitors comparing the results obtained by this association and those obtained with G-CSF alone. We enrolled 15 patients aged from 14 to 57 years (median 46); 7 were male and 8 female; 8 were affected by high-grade non-Hodgkin's lymphoma, 2 by Hodgkin's disease, 3 by non hematologic malignancies, 1 by multiple myeloma and 1 by acute lymphoblastic leukemia. Most of the patients had been previously heavily treated with a number of chemotherapy courses ranging from 3 to 30 (median 6); only one patient had received previous radiotherapy. Six patients were in complete remission, 8 in partial remission and one in sensitive relapse; 2 patients had bone marrow involvement. The first group of 7 patients after high-dose chemotherapy received G-CSF 5 µg/kg/die, while the second group of 8 patients were given G-CSF at the same dose plus EPO 4000 U/die until leukapheresis were stopped. The two group were homogeneous for the main clinical and hematological characteristics. As for the stem cell mobilization data, we observed better results in the group treated with G-CSF plus EPO, with a mean increase of 1.8 fold for circulating MNC, 1.6-fold for CFU-GM, 3.1-fold for BFU-E, 1.3-fold for CFU-GEMM and 0.8-fold for CD34+ cells. As regarding the results of collections from 48 leukapheresis, the addition of EPO showed an impressive increase not only in terms of MNC (1.8-fold) but also concerning the hemopoietic progenitors: CFU-GM (3.9-fold), BFU-E (4.3-fold), CFU-GEMM (1.2-fold) and in particular in terms of CD34+ cells harvested (5-fold). The difference was statistically significant for CFU-GM/kg (p=0.0172) and for CD34+/kg (p=0.0004) collected per leukapheresis. Our data suggest that EPO has a synergistic activity with G-CSF in mobilizing multipotential hemopoietic progenitors; the good results, obtained despite the most of patients was heavily pretreated, suggest that this association could have clinical relevance: indeed, in all patients primed with G-CSF plus EPO the amount of CD34+ cells/kg harvested per single leukapheresis (mean = 7.6×10^6/kg; IC95%=4.4-10.8) exceeds the acknowledged effective threshold dose for rapid and sustained engraftment.

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SEQUENTIAL ADMINISTRATION OF INTERLEUKIN-3 (IL-3) AND GRANULOCYTE-MACROPHAGE COLONY-STIMULATING FACTOR (GM-CSF) AFTER HIGH-DOSE CYCLOPHOSPHAMIDE (HDCY)

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High-dose cyclophosphamide (HDCY) is an effective treatment for a variety of solid tumors including Hodgkin's disease, non-Hodgkin lymphomas, breast cancer and multiple myeloma and is incorporated with increasing frequency in modern chemotherapy programs. The addition of hemopoietic growth factors, mainly granulocyte colony-stimulating factor (G-CSF) and GM-CSF to HDCY reduces the risk of neutropenic infection and of bleeding complications and improves the recruitment of peripheral blood progenitor cells (PBPC). Here we report on the toxicity and the effectiveness of IL-3 and GM-CSF sequentially administered after HDCY. Twelve patients (pts) with poor risk breast cancer (8) or non-Hodgkin lymphoma (4), aged 32 to 50 years (mean 42.4) were enrolled. Most pts (8) had received previous chemotherapy, and one presented with bone marrow involvement. HDCY (6-7 g/sqm) was given on day 0 in five divided doses, supported by the sequential administration of IL-3 (250 mcg/sqm/d) from day +1 to +7 and GM-CSF (5-7 mcg/Kg/d) from day +7 to +14 (or to neutrophil recovery). At the time of hematological recovery (WBC > 1000/mcL, platelets > 50000/mcL) the pts underwent 4 leukapheres for PBPC collection. PBPC were defined as cells expressing the surface membrane protein CD34 by direct immunofluorescence analysis. The leucocyte recovery was fast and all pts achieved an absolute neutrophil count (ANC) more than 1000 and more than 2500/mcL on a mean of 13.4 (range 12-15) and 14.0 (range 12-16) days respectively. The high risk neutropenia (ANC < 100/mcL) and severe neutropenia (ANC < 500/mcL) were of brief duration, with a mean of 3.6 (range 0-6) and 6.2 (range 4-9) days, respectively, and no documented infection was observed. In no pts the platelet (plts) count fell below 20000/mcL and in one case only below 50000. No prophylactic plt transfusion was required. During IL-3 administration 9 pts experienced headache slight to moderate, 5 pts fever and 4 pts erythematous skin rash. GM-CSF infusion was associated to skin rash in 7 pts, mild osteoarticular pain in 4 pts and mild pruritus in 4. No pts required growth factor discontinuation or dosage reduction. The apheretic procedures yielded a mean of 28.4×10^6/kg CD34 positive cells (range $7.1\text{-}61.9\times10^6$/kg). It is concluded that HDCY followed by the sequential administration of IL-3 and GM-CSF is a fairly well tolerated procedure and allows the recruitment of large numbers of PBPC.

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CORRELATION BETWEEN THROMBOMODULIN (TM) PLASMA LEVEL AND PERIPHERAL BLOOD CD34+ CELL NUMBER AFTER HIGH DOSE (HD) CHEMOTHERAPY

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The application of peripheral stem cell trasplantation (PBSCT) has been used with increasing frequency over the last decade, especially in hemopoietic malignancies and breast cancer. The most updated method involves priming the patient with HD chemotherapy followed by stimulation with G/GM-CSF. It has been shown by Shirota et al. (*Exp Hematol 1991*) that administration of HDCY causes vascular endothelium cell damage allowing this way the traffic of transplanted stem cells to hemopietic compartment.

In keeping with this theory which considers the endothelial cell damamge the key event for stem cell homing, in the present study we tested this hypothesis to verify whether vascular endothelial damage permits stem cells traffic also in the opposite way from bone marrow niches to peripheral blood stream.

To this aim we tried to correlate the endothelial damage evaluated by plasma thrombomodulin increase with the CD34+ cells rise in the peripheral blood. Thrombomodulin tests were performed at basal conditions, on every other day after HDCY or Epi and on the same days of the apheresis.

Our preliminary data suggest a concurrent increase of thrombomodulin, ANC and CD34+ hemopoietic precursors,particularly evident in patients primed with HDCY. With epirubicin instead our data showed a lesser increase of CD34+ cells together with unchanged plasma levels of TM. Should these data be confirmed in a larger number of cases, then a more relevant endothelial damage caused by cyclophosphamide compared to anthracycline would be hypothesized.

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IN VITRO EXPANSION OF CD34+ HEMOPOIETIC STEM CELLS

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Experiments of selection and expansion of hemopoietic stem cells were carried out using cells obtained from normal bone marrow (n=4) and leukapheresis products from non-Hodgkin lymphoma patients receiving chemotherapy plus G-CSF (n=2). Positive selection of CD34+ cells was performed using an avidin-biotin immunoaffinity system (Ceprate LC, CellPro). Liquid cultures to expand CD34+ cells were established in long-term culture medium up to 28 days at 37°C in 5% $C0_2$. The following hemopoietic growth factors and cytokines were used: IL1 (10 ng/mL), IL3 (20 ng/mL), IL6 (25 ng/mL), SCF (100 ng/mL), IFN-γ (100 ng/mL) and EPO (4 U/mL). We obtained an average 47-fold CD34+ enrichment. After 14 days in culture, the BM CD34+ cells declined from an initial mean of 68±4% to 0.37±0.02%, while on PB samples the decrease was from 55.3±4% to 0. The BM CD34+ enriched samples contained significant a fraction of CD34+CD19+ (19.2±9%) that did not proliferate under these culture conditions, while on leukapheresis products no pre-B lymphocytes were observed. During culture, CD33, CD15 and CD11b antigens were sequential expressed, both on bone marrow and peripheral blood CD34+ cells. An exponential increase of both total nucleated cells (163±46-fold) and CFU-C occurred. A peak of CFU-GEMM (9.2±3-fold) and BFU-E (10±2.4-fold) occurred at days 5 and 7 respectively, while a 6.6±1 fold-increase of CFU-GM was observed at day 7, with a plateaux until day 21. In our experiment we observed an increase of early progenitors until day 7 followed by a maturation which gave rise to CD15+ CD11b+ phenotype representing neutrophil forms.

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EXPANSION OF EARLY HEMATOPOIETIC PROGENITORS FROM LEUKAPHERESES WITH A COMBINATION OF FOUR CYTOKINES WITHOUT CD 34+ CELLS PURIFICATION

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During the last years many attempts have been made in order to expand the hematopoietic stem cells, both from bone marrow and from peripheral blood. Involving the purification of CD34+ cells. This technique is time-consuming and does not lead to good recovery (about 35-50%); moreover, regardless from the stem cells source, the different techniques utilized for this aim are characterized by a wide range of results in terms of CD34+ purity (from 60 to 90%). We studied 5 samples of mononuclear cells (3 fresh and 2 cryopreserved) from peripheral blood in a long term liquid culture system in the attempt of expanding the hemopoietic progenitors without previous CD 34+ cells purification. The mononuclear cells, obtained from 5 leukaphereses in 3 patients primed with chemotherapy followed by G-CSF, were seeded in duplicate, in two different media at the concentration of 2.5×10^6/mL: IMDM plus Fetal Calf Serum without growth factors and the same medium plus four cytokines: IL1, IL3, IL6, SCF. The culture flasks have been weekly demipopulated and refed. Cell count, viability by trypan bleu exclusion, clonogenic assay in semisolid medium (MEC) and immunophenotype have been checked weekly until the 5th week of liquid culture. Only the addition of the four cytokines showed to induce an expansion of the hematopoietic progenitors while in the system without cytokines we observed a progressive decrease of cellularity and of colony output. In the system enriched with the cytokines we observed the peak of cellularity (2.8-fold) at the 4th week, even if the hematopoietic progenitors (CFU-GM) peacked at the 3th week of liquid culture (11 folds the baseline values), followed by significant drop (5.8 folds the baseline values) at the 4th week. The phenotype of leukaphereses changed significantly after four weeks with a reduction of CD2+ cells (from 33±30% to 16±17%), an increase of CD19+ (from 9.5±16% to 45±27%) and of CD14+ (from 37±20% to 58±20.5%). The percentage of the CD34+ cells decreased from 4.8±5.7% to 3.76±3.7%, but the absolute number of CD34+ cells resulted increased after 4 weeks (two folds). Finally we observed the drop of BFU-E output at the second week of culture, probably because of the absence of erythropoietin in the medium.

These preliminary data support the possibility to expand the peripheral hematopoietic progenitors even without previous CD34+ cells purification; the increase of the CFU-GM output (5.8-fold) after four weeks of culture suggests that this expansion significantly involves also the early progenitors. The best results are obtained at the 3rd week, but without expansion of the erythroid precursors; further investigations are needed in order to evaluate the expansion of stem cells and to establish if the EPO addition can improve these results.

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ANALYSIS OF HEMATOPOIETIC PROGENITOR CELLS FROM UMBILICAL CORD BLOOD AFTER 3 WEEK SUSPENSION CULTURE WITH DIFFERENT GROWTH FACTORS

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The progenitor number contained in a sample of umbilical cord blood (UCB) is not considered permissive for engraft an adult patient. The aim of the study was to value the persistence and the possibility of increasing the UCB proliferative activity in liquid culture for 3 weeks, with or without different concentrations of growth factors. One$\times 10^6$ monuclear cells from 10 samples of UCB were cultured in 25 mL flask (Falcon) in 10 mL Iscove Modification of Dulbecco's Medium (GIBCO) supplemented with foetal calf serum 20% (Seromed), hydrocortisone 10^{-2} Mol, GM-CSF (100 ng/mL). Every sample were cultured without growth factors and with IL3 (100 U/mL), GM-CSF + IL3 at the same concentrations. Refeeding were performed every 7 days. At 7, 14, 21 days the cell contained in the supernatant were cutred in methylcellulose for CFU-GM, BFU-E and CFU-GEMM. The results are reported in Table 1.

week	*standard*			*GM-CSF 100 ng/mL*			*IL3 100 U/mL*			*GM-CSF 100 ng/mL + IL3 100 U/mL*		
	A	B	C	A	B	C	A	B	C	A	B	C
0	27.1	71.4	24									
1	61.2	11.4	8.2	80.5	16.3	2.7	73	19.1	6.7	97.7	34.8	10.7
2	11.1	1.4	1.4	12.5	2.8	1.7	36.5	6.5	6.2	11.1	0.5	2
3	2.8	0.4	0.4	2.8	0.8	0	21.6	0.8	4	2.4	0.4	0

A= CFU-GM /10^5 cells, B= BFU-E /10^5 cells, C=CFU-GEMM /10^5 cells

After 7 days the plating efficiency of CFU-GM, BFU-E and CFU-GEMM was substantially the same of the standard controls (in presence of IL3+GM-CSF was scored a moderate increase of BFU-E and CFU-GEMM numbers but without statistic significance) The proliferative activity fells progressively after 14 and 21 days of culture. A moderate persistence of BFU-E and CFU-GEMM was observed in presence of IL3. In conclusion according to our results seems that is not possible to maintain a satisfactory hemopoiesis with the culture system utilized, still in presence of growth factors. However, IL3 was able to sustain the growth of some undifferentiated progenitors. It is possible that combinations of many growth factors and the enrichment of the medium culture could ameliorate the UCB progenitors cell growth.

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PROLIFERATIVE ACTIVITY OF HEMATOPOIETIC PROGENITOR CELL FROM UMBILICAL CORD BLOOD (UCB) WITH IL3 AND GM-CSF

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Cord blood (UCB) has been utilized successfully for the hemopoietic reconstitution of children with disorders of hematopoiesis as an alternative to marrow derived stem cells. However, the most significant limitation to the wide use of UCB in adult transplant may be the number of precursors that can be obtained. Previous results suggest that the total number of progenitors in a cord blood sample, harvested from one placenta may be to low to engraft an adult recipient. Therefore we preculture for 7 days in liquid suspension the UCB with GM-CSF (10 and 100 ng/mL) and IL3 (10 and 100 U/mL) and than we check the content of the progenitor cells (CFU-GM, BFU-E, CFU-GEMM). The results are reported in Table 1.

	CFU-GM$\times 10^5$ cell (SD)	BFU-E$\times 10^5$ cell (SD)	CFU-GEMM$\times 10^5$ cell (SD)
Standard	63.6 (51)	89.2 (100)	28 (28)
After 7 d. of liquid culture	79.7(51)	56 (46)	51.7 (49)
GM-CSF 10 ng/mL	102 (20)	102 (39)	101 (15)
GM-CSF 100 ng/mL	145 (113)	84 (78)	46 (43)
IL3 10 U/mL	122 (112)	100.6 (100)	50.8 (50)
IL3 100 U/mL	93.7 (46)	78 (50)	63 (46)
GM-CSF 10 ng+ IL3 10 U/mL	100 (18)	97 (23)	96 (22)
GM-CSF 100 ng+ IL3 100 U/mL	110 (67)	57 (45)	55 (47)

Table 1. Hematopoietic progenitor cell growth in standard controls and after incubation for 7 days with or without IL3 and GM-CSF. The results are expressed as mean (SD) of 20 samples.

The UCB cells seem to maintain their proliferative potential, without CSF, after 7 days of suspension culture. According to our data GM-CSF is probably able to increase the growth of CFU-GM while IL3 seems to stimulate a kind of cells more immature. Further increase of the proliferative activity was not observed with the association of the two growth factors.
In conclusion our data confirm the high proliferative potential of UCB hematopoietic progenitor cells. The results obtained do not allow conclusive report about the capability of GM-CSF or IL3 to rise the progenitor number in UCB samples but they suggest the possibility that this goal could be reached with different doses of the two growth factors here tested, eventually associated with other cytokines such as the stem cell factor.

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IDENTIFICATION AND CHARACTERIZATION OF ERYTHROPOIETIN RECEPTORS ON THE HUMAN HEP3B HEPATOMA CELL LINE

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The human Hep3B hepatoma cell line is a well characterized *in vitro* system for studies of hypoxia-induced erythropoietin (Epo) production, since these cells have been shown able to modulate Epo mRNA levels in response to an hypoxic stimulus.
By using reverse-transcription and PCR amplification, we found that unstimulated Hep3B cells also expressed Epo-R mRNA in addition to Epo mRNA. The presence of functionally competent Epo-R molecules was demonstrated by binding studies using either biotinylated Epo (analyzed by fluorescence activated cell analyzer and by in-situ cytochemistry) or iodine-labelled Epo; the number of receptors was about 500-1000/cell, and only a low-affinity Epo-R class was found. The incubation of normoxic Hep3B cells with different amounts of Epo had no effect on both the proliferative (as measured by tritiated thymidine incorporation) and the protein synthetic rate (as measured by tritiated aminoacid incorporation).
In order to verify the possible role of Epo-R in the modulation of hypoxia-induced Epo mRNA expression, cells exposed to hypoxia (1% O_2) were cultured in the presence of varying amounts of exogenously added Epo.
The effects of exogenous Epo on the levels of Epo mRNA expression were evaluated by a quantitative PCR method, based on a scintillation proximity assay of tritiated PCR products captured by an Epo-specific biotinylated primer; normalization of Epo mRNA levels was done in comparison with the relatively invariant β-actin mRNA levels. Preliminary data indicate little effects of exogenous Epo on its own production by hypoxic Hep3B cells.
In conclusion, the finding of Epo-R on cells producing Epo has not been previously reported; however, the functional relevance of Epo-R on these cells remains to be ascertained.

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FLOW CYTOMETRY DETECTION OF GM-CSF-R IN ACUTE MYELOID LEUKEMIA AND MYELODISPLASTIC SYNDROMES

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Cell reactivity for the GM-CSF receptor was assessed by flow cytometry in 10 AML, 10 MDS, and ten healthy subjects, using two monoclonal antibodies that recognize the α-subunit of the receptor. The results indicate that both neutrophils and monocytes from the peripheral blood of normal subjects express a significant amount of GM-CSF-R (median MESF values: 3,368, and 5498, respectively), while the same cells taken from either MDS or AML exhibited both qualitative and quantitative deficiency in GM-CSF-R expression. Furthermore, though CD34+ bone marrow progenitors cells from healthy subjects react with GM-CSF-R in all the cases examined (median MESF values: 9,850), blasts from AML were GM-CSF-R positive in only 33% of the cases, and cell positivity seem to be restricted to early FAB subtypes (M0-M1), and was associated with expression of CD34, HLA-DR and CD38 surface markers. A preliminary analysis of AML patients did not show any correlation between GM-CSF-R expression and clonogenic characteristics of blasts' cells. In conclusion, the correct application of a flow cytometry technology in combination with the use of standard calibration microbeads would allow a precise and reliable quantitation of either molecular equivalents of soluble fluorochrome (MESF) or the number of binding sites for GM-CSF-R per cell, allowing also the possibility to compare the flow cytometry data over time and between one laboratory and another. The cytofluorimetric determination of GM-CSF-R in AML blasts further offer the possibility to select patients suitable for GM-CSF treatment following intensive myeloablative chemotherapy regimens, avoiding the risk of stimulating the growth of blast cells.
The possibility of using GM-CSF in AML patients who do not express GM-CSF-R represents another fascinating field of application of this approach, which would permit the recruitment of blast cells into active phases of cell cycle prior to chemotherapy, with the aim of increasing cell killing and consequently remission rates of these patients.

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EFFECTS OF THE c-kit LIGAND AND OTHER GROWTH FACTORS ON BLAST CELL PROLIFERATION OF ACUTE MYELOID LEUKEMIA PATIENTS: SYNERGISTIC INTERACTION BETWEEN SCF AND PIXY 321 AND ENHANCEMENT OF ARA-C CYTOTOXICITY

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Priming of acute myeloid leukemia (AML) cells with growth factors (GF) has been reported to increase *in vitro* percentage of cycling cells and enhance cytotoxic effects of chemotherapeutic agents through either kinetic or other mechanisms. Heterogeneity of proliferative response to different GFs may be taken into account for minor clinical benefit in AML. Therefore in this study we have compared *in vitro* proliferative effects of several GFs on 20 *de novo* AML samples. Stem cell factor (SCF) as well as G-CSF, GM-CSF, IL-3, GM-CSF/IL-3 fusion protein (PIXY 321) and their combinations were used in liquid colture for 48-72 hours to determine both proliferative effects on clonogenic cell growth (CFU-L), and cell cycle changes by flow cytometric DNA/RNA (acridine-orange).
Expression of SCF receptor, the c-kit protein, was also measured by flow cytometry and correlated with proliferative response. AML blasts, after GF priming, were then exposed to different concentrations of Ara-C and cytotoxicity was measured as percentage of CFU-L inhibition.
Results showed a significant recruitment into the cell cycle induced by SCF as demonstrated by a mean decrease of G0 from 50% to 33.6 (p=0.002) paralleled by an increase in S-phase (from 6.8% to 15.8%, p=0.0002). A 2.4 fold increase was also found in the clonogenic cell growth. The combination of SCF+PIXY induced an higher proliferative response than other GFs. Heterogeneity in GF response were found among the cases analyzed. Cytotoxic effect of Ara-C were enhanced especially by SCF+PIXY, although benefit in leukemic cell killing was also different from case to case.
In conclusion this *in vitro* study provides further observation useful for the optimal utilization of cytokines, as priming molecules, in the AML treatment.

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EFFECTS OF SCF, IL-3 AND ERYTHROPOIETIN ON THE DIFFERENTIATION OF A HUMAN ERYTHROLEUKEMIA CELL LINE (TF-1).

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TF-1 is an established human erythroleukemia cell line, which constitutively expresses receptors for Epo, IL-3 and GM-CSF, and showes a growth dependency on these factors. Stem cell factor (SCF) was also shown to be able to induce the proliferation of TF-1 cells. Although the cells are benzidine and glycophorin negative, morphology and some cytochemical marker (PAS positivity) suggest that they are erythroid in origin. Moreover the line expresses globin genes and synthesizes HbF in response to inducers such as Hemin or δ-ALA. Finally a single class of high affinity Epo receptors (Epo-R) has been described. Very recently it was found that short term (16 hours) incubation of cells with IL-1a plus Epo, but not with other cytokines or Epo alone, increases Epo-R mRNA (*Winter et al. Blood 82, Supp 1, p 227a, 1993*). The purpose of this study was to establish the effectiveness of erythropoietin, SCF and IL-3 in inducing further differentiation of TF-1 into the erythroid pathway. Benzidine staining remained negative after incubation of TF-1 in the presence of each factor, while glycophorine was weakly expressed only in the presence of SCF. To evaluate Epo-R expression, which in normal erythropoietic cells is maximally expressed at the CFU-E level, cells were acid-washed to eliminate the surface-bound Epo, and were analyzed by flow cytometry after amplified immunostaining with biotin-labelled Epo, streptavidin-conjugated R-phycoerythrin, and biotinylated monoclonal anti-R-phycoerythrin (*Wognum et al., Blood 79, 642; 1992*). An increased expression of Epo-R was evident in the cells incubated with Epo for five days and, to a lesser extent, in those treated with the combination of Epo+SCF for the same time, but not in the cells incubated with IL-3 or SCF alone. In other experiments carried out with ^{125}I Epo, binding of radiolabeled Epo to cells was found increased by the preincubation with Epo if compared to unstimulated cells. Moreover, the Scatchard analysis suggested that, instead of the single class receptor observed in the absence of Epo, incubation with Epo causes the cells to express two classes of receptors, a feature of normal human erythroid progenitors.
Binding sites and kd values were calculated to be respectively 648±31 and 0.233±0.03 for the high affinity component; 2568±78 and 0.920±0.05 for the low affinity component.

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TGF-β3 INHIBITS HUMAN PRIMITIVE HEMATOPOIETIC PROGENITORS. IN-VITRO EFFECTS OF THE EARLY ACTING CYTOKINES IL-11, SCF AND IL-9

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We have investigated the antiproliferative activity of TGF-β3 on highly purified bone marrow (BM) CD34+ cells and more immature CD34+/4-hydroperoxycyclophosphamide (4-HC) resistant cells. Primitive hematopoietic progenitors were stimulated by early acting colony stimulating factors (CSFs) such as SCF, IL-11 and IL9, and the intermediate-late acting growth factors IL-3 and GM-CSF, alone and in combination.
The addition of TGF-β3 to cultures of CD34+ cells containing IL-11, IL9 or SCF alone resulted in 85% or more inhibition of the total colony formation. Conversely, the IL-3 and GM-CSF-stimulated colony growth was inhibited by 57% and 58%, respectively ($p < 0.02$). IL-11, IL9 and SCF acted synergistically or additively with IL-3 and GM-CSF, in presence and absence of TGF-β3. The percentage of CD34+ cells inhibited by TGF-β3 was significantly reduced when IL-11 and IL9, but not SCF, were added to the other cytokines. Similarly, the colony growth of CD34+ cells in response to IL-11 and SCF in combination was inhibited by TGF-β3 by 52.4% as compared to 90% or more when the same cytokines were used alone. When tested on CD34+/4-HC resistant progenitors, IL-11, IL9 and SCF increased the number of clonogenic cells responsive to IL-3 and GM-CSF. However, TGF-β3 demonstrated on earlier cells a greater inhibitory activity as compared to the more mature CD34+ cell fraction and none of the study cytokines completely abrogated the activity of TGF-β3.
Finally, the results of a two-step liquid culture assay (Delta Assay) showed that IL-11 and SCF or IL-9 and SCF in combination were capable of stimulating the growth of a subset of non-clonogenic pluripotent hematopoietic precursors (pre-CFU-C) which escaped from the inhibitory effect of TGF-β3.
In summary, these data confirm that TGF-β3 exerts its suppressive activity on hematopoietic progenitor cells depending on the differentiation state of the target cells and the other cytokines interacting with the cells. The synergistic growth factors IL-11 and IL-9 seem to be able to partially counteract the negative regulation of TGF-β3.

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TNF RECEPTORS ARE EXPRESSED BY ACUTE LEUKEMIA CELLS AND ARE ASSOCIATED WITH INCREASED SERUM LEVELS OF THE CORRESPONDING SOLUBLE MOLECULES

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TNF receptors (TNFRs) exert a regulatory role of TNF functions not only as cell membrane binding structures, but also as soluble molecules (sTNFRs), released by TNFR-expressing cells, with TNF-binding capability. The TNF/TNFR system is involved in autocrine and paracrine mechanisms of leukemic cell growth. Serum levels of sTNFR-55 and sTNFR-75 were determined by an ELIBA test kit in the sera of 120 patients with acute leukemia at diagnosis without documented infection. Eighty-seven were AML (M0:3 M1:14, M2:18, M3:15, M4:22, M5:10, M6:3, M7:2) and 33 ALL (*common*: 22, T: 8, *null*: 3). Results were as follows:

	controls	ALL	AML	M0-M3	M4-M5
(n° 90)	(n° 33)	(n° 87)	(n° 50)	(n° 32)	
p55*	1.93±0.54	3.99±1.50	4.46±3.19	3.34±3.34	6.09±4.53
p75*	2.30±0.77	6.52±2.89	7.34±6.88	4.67±2.77	10.93±9.01

**ng/mL. Statistics: ALL vs AML: p=ns; MO-M3 vs M4-M5: p<.001 for both chains; ALL and AML vs controls: p<.01 for both chains.*

The presence of high circulating levels of sTNFRs prompted us to study the pattern of membrane expression of TNFR molecules on leukemic cells. Blast cells from 15 cases were investigated, at basal conditions and after 24-hour culture (RPM1+ FCS), with htr-9 (anti-TNFR-55) and utr-1 (anti-TNFR-75) MoAbs by flow cytometry. The majority of cases (AML: 9/10; ALL: 5/5) expressed both TNFR molecules at basal condition. Following culture, an increased membrane expression of both TNFRs was observed: TNFR-55 in 7/15 cases (AML 4/10, ALL 3/5) and TNFR-75 in 7/15 cases (AML 6/10, ALL 1/5). The strongest membrane expression (before and after culture) of both TNFRs was found in M4-M5 AMLs. Soluble TNFRs were present in SN of cultured blasts. The data indicate that sTNFRs are released by leukemic cells in acute leukemia. The increased circulating levels of these soluble molecules might play a functional role in the complex mechanisms of leukemic growth.

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EXPRESSION AND FUNCTIONAL ROLE OF C-KIT LIGAND (SCF) IN HUMAN MULTIPLE MYELOMA (MM) CELLS

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In this study we investigated the proliferation of 3 well-documented MM cell lines and 10 bone marrow samples from myeloma patients in response to rh-SCF alone and combined with interleukin-6 (IL-6), IL-3 and IL-3/GM-CSF fusion protein PIXY 321. Neoplastic plasma cells were highly purified (>90%) by immunomagnetic depletion of T, myeloid, monocytoid and NK cells. The number of S-phase cells was evaluated after 3 and 7 days of liquid culture by the bromodeoxyuridine (BRDU) incorporation assay. The proliferation of RPMI 8226 and U266 cell lines was also assessed by a clonogenic assay. All the experiments were performed in serum-free conditions. RPMI 8226 cell line was not stimulated by SCF which also did not augment the proliferative activity of IL-6, IL-3 and PIXY-321. Conversely, SCF addition resulted in 2.4-fold increase of the number of U266 colonies and in a higher number of U266 and MT3 cells in S-phase (24.5±2% SEM vs 14.5±1% SEM and 32±3% SEM vs 21±4% SEM, respectively; $p < 0.05$). The c-kit ligand also enhanced the proliferation of MT3 and U266 cells mediated by the other cytokines. Anti-SCF polyclonal antibodies completely abrogated the proliferative response of MT3 cells to exogenous SCF and markedly reduced the spontaneous growth of the same cell line. Reverse transcriptase-polymerase chain reaction amplification (RT-PCR) did detect SCF mRNA in MT3 and RPMI 8226 cells. Moreover, secreted SCF was found, in a biologically active form, in the supernatant of the two cell lines by the MO7e proliferation assay.
When tested on fresh myeloma samples, SCF increased the number of S-phase plasma cells (4.7±1.6% vs 3.4±1.3% in control cultures; p=0.02). Significant proliferation was also induced by IL-6 (7±2.3% of BRDU+cells; p=0.006), IL-3 (5.3±1.3%; p=0.01) and PIXY-321 (5.4±1.6%; p=0.02). The addition of SCF significantly enhanced the proliferation of myeloma cells responsive to IL-6.
In summary, our results indicate that SCF is expressed in MM cells and it stimulates the proliferation of neoplastic plasma cells.

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ARACHIDONIC ACID MEDIATES INTERLEUKIN-1 AND TUMOR NECROSIS FACTOR-INDUCED C-JUN EXPRESSION IN STROMAL CELLS BY A PROTEIN KINASE C-INDEPENDENT PATHWAY

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We have previously demonstrated that GM-CSF gene expression induced by IL-1 plus TNF in the murine stromal cell line, +/+–1. LDA 11, involves activation of phospholipase A_2 (PLA_2). Increased expression of the transcriptional factor, c-jun, which is induced in response to release of arachidonic acid by IL-1 and TNF-activated PLA_2, may be important in activation of GM-CSF gene transcription (*Rizzo, Boswell. Exp Hematol 22:87, 1994*).
The present study was undertaken to explore potential mechanisms by which arachidonic acid induces expression of c-jun in ++–1. LDA 11 cells. Treatment of stromal cells with cycloheximide did not inhibit arachidonic acid induced c-jun expression, suggesting that arachidonic acid may induced post-translational modification of the pre-existing c-jun/AP1 complex. Depletion of cellular protein kinase C activity by pretreatment with TPA (400 nM) abolished c-jun expression induced by TPA, but had no influence on c-jun expression induced by arachidonate. In contrast, pretreatment of stromal cells with the tyrosine kinase inhibitor, genistein, decreased in a time- and dose-dependent manner, c-jun expression induced by 50 μM arachidonic acid. Genistein similarly inhibited c-jun expression induced by IL-1 and TNF. Exposure of stromal cells to arachidonic acid induced a 2.6-fold increase in intracellular tyrosine kinase activity, determined by phosphorylation of the syntetic peptide raytide in the presence of 32P-ATP. Similarly, stimulation of stromal cells with IL-1 and TNF induced a 3.2- and 7.2-fold increase in intracellular tyrosine kinase activity, respectively. These results are consistent with the hypothesis that arachidonic acid mediates IL-1 plus TNF-induced expression of c-jun by the intermediate activation of a non-receptor protein tyrosine kinase. This putative enzyme effector may act in a protein-kinase C (PKC)-independent signal transduction pathway to transmit information between plasma membrane growth factor receptors and the nucleus.
We hypothesize that this PKC-independent pathway may involve p21RAS.

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MEMBRANE-BOUND, SOLUBLE IL-2 RECEPTORS (IL-2R) AND LEVELS OF IL-1α, IL-2 AND IL 6 IN PATIENTS WITH HEMATOLOGICAL MALIGNANCIES

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The aim of the present study has been to evaluate: 1) the peripheral blood mononuclear cells (PBMC) blastic responses to PHA, PHA plus recombinant IL2 (r IL 2) and rIL2 alone; 2) the expression of membrane-bound IL2R, the p55 chain (CD25), on PHA-stimulated PBMC (at 3 days); 3) the levels of soluble IL2R (sIL2R) in serum and in culture supernatants from PHA-stimulated PBMC (at 7 days); 4) the levels of IL1α, IL2 and IL6 in serum and in culture supernatants from PHA-stimulated PBMC (at 1 day) in patients (pts) with hematological malignancies.
Seventeen pts have been studied (mean age 58.5 years, range 22-82): 4 Hodgkin's lymphoma (HL), 2 with active disease and 2 in clinical remission; 6 non-Hodgkin's lymphoma (NHL), 4 with active disease and 2 in clinical remission; 5 hairy cell leukemia (HCL); 1 chronic myelogenous leukemia (CML) and 1 chronic lymphocytic leukemia (CLL). Twenty healthy age-sex-matched subjects served as controls. The membrane-bound CD25 was detected by flow cytometry utilizing FITC monoclonal antibody OKT26a, whereas the ELISA test was performed for the detection of sILR and the cytokines.
Our data show that the PBMC blastic response to PHA, PHA plus rIL2 and rIL2 alone was significantly reduced in pts with active disease in comparison to healthy controls. The expression of membrane-bound CD25 on PHA-stimulated PBMC was in the same range in pts, irrespective to the disease state, and in controls. Increased serum values of sIL2R, IL1α, IL2 and IL6 were found in all pts with active disease, whereas the levels of the same cytokines in culture supernatants of PHA-stimulated PBMC were lower than controls. The study is still in progress in order to elucidate the cytokine background underlying the two different clinical patterns, such as the active disease and the remission, respectively.

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EFFECTS OF IL-4 AND IL-7 ON THE GROWTH AND DIFFERENTIATION OF NORMAL B CELL PRECURSORS

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Cellular and molecular studies of human B cell precursors (BCPs) have been hampered by the absence of a reproducible culture system. The fetal liver represents in humans the major source of BCPs and preB cells. We studied the activity of IL-4 and IL-7 on a pre B cell enriched population obtained from fetal liver, by removing non pre B cells with CD7, CD13, CD33, CD11b, R10 and anti-Ig antibodies plus antimouse IgG immunomagnetic beads. The presence of IL-4 receptors on early BCPs have been shown, but studies on the effects of IL-4 on these cells have resulted in somewhat contradictory results. However, IL-4 may have different effects on different subpopulations of BCPs depending on the combinations of different stimuli acting on a given cell. In our work, IL-4 did not induce neither growth nor differentiation of pre B cells. Furthermore it was able to increase pre B cell number and sIg appearance in the presence of contaminant accessory cells, suggesting that stromal cell contact and/or regulatory humoral molecole are necessary to mediate BCPs differentiation. The rabbit anti human IL-4 antibody abrogated the inducing effect of rIL-4 on sIg expression, confirming the specificity of the biological activity of rIL-4. IL-7 induced proliferation of the fetal liver pre B enriched population according to previous reports but did not show differentiation potential.
Finally, IL-7 presented colony stimulating activity on the same cell population. The immunophenotype of IL-7-dependent colony forming cells was determined by immunofluorescent staining. The majority of cells were CD7-, cCD22+, CD19+,CD10+,Tdt+ confirming there B lineage affiliation and B precursor phenotype.
In conclusion, we have demonstrated the IL-4 stromal dependent induced differentiation and IL-7 dependent proliferation and clonogenic potential presented on CD10+, CD19+, Tdt+, sIg–, cIgM+ (stage II BCPs, *Uckun et al. 1992*) population of fetal liver cells at the gestational age ranging from 10 to 14 weeks.
This short term culture model for a fetal liver cell isolated population by immunomagnetic bead depletion allows for further studies on the events regulating human pre B cells growth and differentiation.

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INTERLEUKIN-8 MAY EXERT A FUNCTIONAL AUTOCRINE/PARACRINE ROLE IN B-CELL CHRONIC LYMPHOCYTIC LEUKEMIA BY PROLONGING THE SURVIVAL OF THE NEOPLASTIC CLONE

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Several cytokines have been suggested to play a regulatory action on the neoplastic clone of patients with B-cell chronic lymphocytic leukemia (B-CLL) by interfering in the differentiation, proliferation or death/survival programs in a paracrine or autocrine fashion. Interleukin 8 (IL8) is a chemoattractant protein constitutively produced and released by B-CLL cells.
The detection of IL8 receptor mRNA (in 7/9 cases) and of IL8 specific binding on B-CLL cells (in 10/10 cases) led us to hypothesize a possible functional role for IL8 in the pathogenesis or clinical course of this chronic lymphoproliferative disorder characterized by the accumulation of monoclonal CD5+ B cells. In B-CLL cultures set up in the presence of recombinant IL8 (rIL8) the cytokine failed to show any proliferative effect. By contrast, the propidium iodide staining of the DNA content and the MTT colorimetric test showed that in 8 of the 13 B-CLL samples studied IL8 induced a prolonged survival effect after 48 hours culture. This effect occurred at the dose range of 5-50 ng/mL and in the presence of at least 30% of spontaneously dying cells. It was not seen in the remaining 5 cases in which a viability of more than 85% was maintained. The dose range employed is comparable with the levels of IL8 released constitutively by B-CLL cells. In 4 of 8 B-CLL cases, IL8 sustained viability also during 0.0001M hydrocortisone (HC) treatment: in the remaining 4 cases, HC treatment induced a greater than 70% cell death and under these conditions rIL8 exerted no further effect. The *in vitro* prolonged survival induced by IL8 is reflected by an increased expression of Bcl-2 mRNA in 5 out of 7 B-CLL cases, in comparison to unstimulated or HC-treated cells. Furthermore, IL8 was capable of enhancing the IL8 mRNA expression in 10 out of 12 B-CLL samples, suggesting a potential autocrine control for IL8 on this cell population. On the basis of these findings, IL8 may play an autocrine or paracrine function in B-CLL by preventing the spontaneous or induced death program of the malignant B-cell clone and, thus, by contributing in the B-cell accumulation process characteristic of this disease.

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IN VITRO EFFECTS OF rhIL-2 ON MDS BONE MARROW: ANALYSIS OF CLONOGENIC GROWTH AND CYTOKINES PRODUCTION

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To evaluate the therapeutic potential of IL-2 in myelodysplastic syndromes (MDS) we studied the *in vitro* effects of this cytokine on blasts proliferation in 34 patients (pts) with MDS (9 RA, 5 RARS, 10 RAEB, 4 RAEB-t, 5 CMML). Bone marrow and peripheral blood mononuclear cells (BMMNC) were plated in IMDM with FCS at a concentration of 1×10^6/mL. After one week rhIL-2 200U/mL or 50U/mL was added to the culture and clonogenic activity was evalueted in agar after 24 and 72 hours. The percentage of blasts and cell morphology were determined using a Romanowsky-stained cytospin preparation. The realease of cytokines into the supernatant of these cultures was measured by immuno-bioassay. The peripheral blood cells (PBMNC) were activated *in vitro* with rhIL-2 500U/mL for 6 days, and then cocultured with autologous BM cells (effector-target ratio= 2:1).
Cell morphology and clonogenic activity were evaluated after 18 hours. CD 56-positive PBMNC were evaluated using a cytofluorometer both before and after *in vitro* activation with rhIL-2. All of the pts had normal serum IL-2 levels. After incubation with rhIL-2 clonogenic BMMNC activity at 24 and 72 hours had increased in all FAB subtypes, but this increase was statistically significant only in RAEB and RAEB-t (p=0.01). However, in most cases, the absolute number of blasts had decreased with a stimulation index (SI= N° of blasts after stimulation/N° of control blasts) of <1. At 24 hours, those cases which had not responded to rhIL-2 200U/mL were also unresponsive to 500U/mL. However, at 72 hours, a greater effect of higher rhIL-2 doses was observed, with all cases except one (RAEB-t with 30% of BM blasts) having an SI<1.
Morphological analysis also showed a small increase in the number of lymphocytes and a greater maturation of BM cells in comparison with untreated controls. γ-IFN and GM-CSF were found to be produced in significant amounts (p>0.05) by marrow mononuclar cells during culture in rhIL-2; in contrast α-TNF levels increased but not significantly so. After co-culture, the clonogenic activity of the cells had not changed with respect to controls. The percentage of CD56-positive cells generally increased after rhIL-2 incubation. Morphological evaluation showed a non-significant reduction in the number of blasts.
These data suggest that rhIL-2 may be useful in a large subgroup of MDS pts (RA, RARS, RAEB, CMML) as it reduces the percentage of blasts and increase clonogenic capacity. The therapeutic usefulness of autologous LAK cells was not demonstrated in this study.

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IN VIVO ANTITUMOR ACTIVITY OF ANTI-CD22/RIPs 1 IMMUNOTOXINS AGAINST DISSEMINATED DAUDI B-CELL LYMPHOMA IN SCID MICE

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We used a SCID mouse model of disseminated human B-cell lymphoma for preclinical evaluation of immunotoxins (ITs) obtained by linking the anti-CD22 monoclonal antibody (mAb) OM124 to the ribosome inactivating proteins of type 1 (RIPs1) Saporin (SO6), Momordin (Mom) and Pokeweed anti-viral protein (PAP). In this model, a single intravenous injection with 5×10^6 Daudi cells induces disseminated B-cell lymphoma which infiltrates lymphoid organs and many extranodal sites, leading to death at day 39.2 after tumor challenge.
The OM124/SO6 IT given intraperitoneally on days 1, 4 and 7 after cell inoculation (total dose: 50% of the LD50 as SO6) showed powerful antitumor activity, delaying the mean survival time (MST) to 50.7 days (p=0.0001). We next compared the therapeutic efficacy of a single IT treatment with CD22/MOM versus a sequential treatment using anti-CD22 coupled to antigenically non cross-reacting ITs (OM124/SO6, OM124/MOM, OM124/PAP) given at three weeks interval. In each cycle of treatment, the above ITs were administered at a dose corresponding to 50% of the LD50. Mice receiving PBS or anti-CD22 mAb alone served as negative control. Both treatment schedules showed significant antitumor activity extending MST by 59.1 and 72.6 days respectively, as compared with either PBS-treated (MST=39.2 days, p=0.0001) and OM124-treated mice (MST=44 days, p=0.0001).
Notably, sequential treatment with three ITs was significantly more effective than a single-course treatment with CD22/MOM (p=0.0004). We conclude that CD22/RIPs 1 ITs are effective anti-tumor agents, sequential administration of antigenically non cross-reacting anti-CD22/RIPs 1 IT being more active than treatment with a single anti-CD22 IT.
Preliminary clinical data strongly suggest that repeated cycles of treatment with anti-CD22/RIPs1 can be also administered safely in humans.

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OVER-UTILIZATION OF IMMUNOGLOBULIN VH4 GENE FAMILY IN AIDS-RELATED NON-HODGKIN LYMPHOMA (AIDS-NHL)

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AIDS-NHL derive from B cells and develop in about 5-10% of AIDS patients with a relative risk of 60 in relation to general population. AIDS-related lymphomagenesis is thought to procede through two main phases. In the first phase host predisposing conditions including highly disturbed immunosurveillance, infection by Epstein-Barr virus and chronic antigen stimulation of the B-cell compartment would cause a polyclonal proliferation of B cells. On this basis multiple genetic lesions would accumulate within one single clone, inducing its neoplastic transformation. Small non-cleaved cell lymphoma (SNCCL) represents one of the two main histotypes of AIDS-NHL.
Previous data have shown that SNCCL AIDS-NHL cell lines secrete immunoglobulins directed against a self-antigen (human actin and the i/I determinants on red blood cells). Furthermore these cases show somatic mutations in the rearranged V_H and V_L genes, consistent with a process of selection by antigen stimulation. These data suggest that self-antigens might play a role in the clonal B-cell expansions that precede neoplastic transformation. To investigate this aspect of AIDS-related lymphomagenesis we have studied the distribution of the V_H family genes utilized by AIDS-related SNCCL (n=8) in order to see whether the distribution is proportional to the relative representation of each gene family in the germline V_H repertoire or if there is an over-utilization of one or more V_H type. Toward this aim we have applied a *half nested* PCR to amplify AIDS-NHL VDJ rearrangements utilizing primers annealing to framework II and JH regions, that enabled us to universally amplify VDJ rearrangements. The PCR product was subsequently gel-purified and directly sequenced to identify the belonging V_H family of the V_H utilized gene.
We have found that 3/8 (37.5%) SNCCL AIDS-NHL have rearranged V_H genes belonging to V_H4 family, 4/8 (50%) belonging to V_H3 family and 1/8 (12.5%) to V_H1 family. When our results are combined with data from the literature we conclude that out of a total of 12 AIDS-NHL tested, 6/12 (50%) utilize V_H4, 5/12 (41.7%) V_H3 and 1/12 (8.3%) V_H1.
In conclusion, V_H gene family usage by AIDS-related SNCCL seems to show a preferential usage of the V_H4 family, since V_H4 genes, which represent only 10% of the total V_H4 repertoire, were found at a significantly higher than expected frequency. The preferential usage of V_H4 by AIDS-NHL is intriguing, since V_H4 is also preferentially utilized by autoreactive B-cell clones in other clinical settings. Our data combined with others from literature suggest that a process of selection and chronic B cell stimulation by a self antigen may be involved in AIDS-related lymphomagenesis.

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ANALYSIS OF BCL 2 EXPRESSION IN NEOPLASTIC AND REACTIVE SKIN LESIONS: A HINT TO THE PATHOGENESIS OF CUTANEOUS T CELL LYMPHOMA?

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Bcl-2 oncogene encoded protein has been shown in a variety of B cell and T cell neoplasias as well as in normal lymphoid tissue where it prevents programmed cell death. However, this oncoprotein has not been thoroughly evaluated in cutaneous T cell lymphomas (CTLC) and related disorders.
It has been recently demonstrated that bcl-2 can play some role in the survival of memory T cells (CD45RO+). Since the majority of CTCL display a memory helper phenotype (CD4+/CD45RO+), we investigated bcl-2 expression in skin biopsies obtained from 12 mycosis fungoides, 10 parapsoriasis, and in 2 cases of primary T cell non-Hodgkin lymphomas (NHL) of the skin. The analysis was carried out, by immunohistochemical methods, on frozen tissue sections using several monoclonal antibodies. In most of the cases T-cell infiltration displayed the common phenotype CD3+/CD4+/βF1+/CD45RO+; one of the T-cell NHL expressed only the γ/δ T cell receptor (TCRδ1+/δTCS1–/βF1–/αF1–) in the context of an immature phenotype (CD3+/CD4–/CD8–/CD1+).
Analysis of bcl-2 showed that in mycosis fungoides as well as in primary NHLs, it is expressed at high level on neoplastic lymphocytes, while the same positivity is detected less intensely in parapsoriasis; in all the cases bcl-2 level correlates with CD25 molecule expression and Ki-67 staining. An indirect piece of evidence that bcl-2 may play some role in the pathogenesis of these skin diseases derives from the inhibition of its expression by drugs that have been recently proposed for the treatment of CTCL (interferon and Tp-5).
Immunophenotypic analysis was performed also on 2 cases of non-neoplastic dermatitis (eczema and lichen) and results showed that bcl-2 protein is detected at high level despite the low expression of CD25. The results of TCR receptor gene rearrangement analysis suggest a hypothesis about the pathogenesis of CTCL in the context of a chronic antigenic stimulus.

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APOPTOSIS INDUCTION WITH THREE NUCLEOSIDE ANALOGS OF FRESHLY ISOLATED B-CHRONIC LYMPHOCYTIC LEUKEMIA CELLS

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The managements of chronic lymphocytic leukemia has remained static for many years. The only active agents were alkylating agents especially chlorambucil and cyclophosphamide alone or with corticosteroids. Recently, three nucleoside analogs, fludarabine (FLU), 2-chlorodeoxyadenosine (2-CdA), and deoxycoformycin (DCF) have showed promising therapeutic activity in the clinical treatment of previously treated and untreated patients with B-chronic lymphocytic leukemia (B-CLL). Recently, some *in vitro* reports have showed the effective role of FLU and 2-CdA on the activation of apoptosis.
The cytotoxic effects and the induction of programmed cell death by FLU, 2-CdA, and DCF with/without α-interferon (α-IFN) were evaluated *in vitro* against freshly isolated B-CLL cells. Cytotoxicity was evaluated according to the soluble tetrazolium/formazan assay. Treatment with FLU and 2-CdA alone or in combination with α-IFN resulted in a remarkable anti-tumor activity against all the samples. On the contrary, a moderate cytotoxic activity was observed with DCF alone or combined with α-IFN, and α-IFN alone showed a very low cytotoxicity. Apoptosis was evaluated morphologically, by electrophoresis gel of DNA oligonucleosomal fragments and by a cytofluorimetric method: only FLU and 2-CdA activated the apoptosis and DCF showed a minor apoptotic pathway increase. FLU and 2-CdA show activity in B-CLL cells by direct cytotoxic action and the induction of cell death by apoptosis; in the future, it would be interesting to utilize these *in vitro* assays in monitoring chemosensitivity and predicting response for the clinical use.

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IN VITRO STUDY OF CHLORAMBUCIL- AND PURINE ANALOGS-INDUCED CYTOTOXICITY IN CHRONIC LYMPHOCYTIC LEUKEMIA. CORRELATION WITH HEMATOLOGICAL FEATURES

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Samples from 55 untreated and 37 treated CLL patients were *in vitro* tested for sensitivity to chlorambucil (CLB), fludarabine (FAMP) and 2-chlorodeoxyadenosine (2-CDA) by a MTT assay. No significant change in terms of ID50 (drug concentration needed to kill 50% of cells) values was seen when results were computed by previous treatment. The wide variability of *in vitro* drug sensitivity allowed us to group samples on the basis of one log increase of ID50 values. According to this procedure, six levels of resistance were identified.
Excluding the last two groups, considered resistant, 50, 35 and 14 samples out of 72 were sensitive to CDA, FAMP and CLB, respectively. Thus CLL lymphocytes consistently exhibited more sensitivity to CDA. However, when samples were defined sensitive to each drug by an ID50 cut-off value corresponding to therapeutically achieviable plasmatic level, FAMP showed the highest cytotoxic effect in both untreated and treated cases. In order to test a possible clinical significance of the MTT assay we compared the *in vitro* drug sensitivity with the *in vivo* clinical efficacy of the same drug in 10 previously untreated CLL cases. The only 2 cases who achieved a complete response after CLB treatment, displayed ID50 values (9.6 μM and 7.5 μM) closed to the therapeutic concentration.
Finally, the correlation between clinical and phenotype features and drug sensitivity has been evaluated by a Pearson's test. Among surface markers, CD11c and CD14 expression significantly correlated with purine analogs' ID50 values in untreated and treated group, respectively. TTM score and bone marrow hystological pattern correlated with the ID50 values of CDA and with the ID50 values of all drugs in treated group, respectively.

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CLINICAL SIGNIFICANCE OF CIRCULATING SOLUBLE ANTIGENS (sCD4, sCD8, sCD23, sCD25, sCD3O, sCD54), A NEW CLASS OF TUMOR MARKERS, IN CHRONIC LYMPHOPROLIFERATIVE DISORDERS (CLD): THE SIX-YEAR EXPERIENCE OF A SINGLE CENTER

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Serum levels of soluble molecules representing fragments of cell specific membrane antigens were monitored, by means of immunoenzymatic assayes, in 580 patients with CLD during the period 1989-94.
One-hundred seventy four patients had non Hodgkin lymphoma (NHL), 80 Hodgkin's disease (HD), 96 chronic lymphocytic leukemia (CLL), 12 Waldenstrom macroglobulinemia (WM), 8 hairy cell leukemia (HCL), 42 MGUS and 168 multiple myeloma (MM). The results obtained by about 3,500 single measurements show that:
a) sCD4 and sCD8 may be useful in monitoring the activity of T-CLD with CD4+ or CD8+ phenotype, respectively; high levels may be detected in various other types of CLD, but without a clear correlation with response to therapy or overall survival;
b) sCD25, sCD3O and sCD54 are interesting markers in HD (sCD30 also in Ki1+ NHL), where their levels at diagnosis correlate with tumor burden and may have prognostic relevance on survival. In particular, increased serum levels of these molecules in remission phase are correlated with early relapses, and this is of particular interest in patients with residual mediastinal masses of uncertain origin after therapy. sCD25 may be found often increased in other CLD, but the eventual clinical significance in this setting, with the well known exception of HCL, is unclear and, in NHL, largely depends on the histologic subtype and treatment applied. In CLL very high levels of sCD25 and/or sCD54 at diagnosis have been found in a subgroup of patients with aggressive disease and short survival. Some MM patients presenting with increased sCD54 also show poor prognosis;
c) sCD23 is a powerful prognostic factor in CLL, strictly related to the extension of the disease at diagnosis and to survival. Low levels of sCD23 characterize instead most of MM patients with active disease or unstable remission, while normal values are usually found in MGUS and in MM with stable disease or *true* plateau phase.

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DISAPPEARANCE OF PCR-DETECTABLE LYMPHOMA CELLS IN PERIPHERAL BLOOD AND BONE MARROW CELL HARVESTS AFTER HIGH-DOSE SEQUENTIAL CHEMOTHERAPY

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A major concern in autografting programs is that peripheral blood (PB) and/or bone marrow (BM) cell harvests used to reconstitute the hematopoietic functions contain occult malignant cells. This is of particular concern in low and intermediate grade non-Hodgkin's lymphomas (NHL) where BM infiltration is common at the time of diagnosis and relapse. Evidence from studies on leukemias and lymphomas support the notion that residual tumor cells contribute to relapse. In the present study, the presence of residual lymphoma cells has been evaluated in PB and BM cell harvests after high-dose sequential (HDS) chemotherapy. Twenty-four patients with low or intermediate grade B-cell NHL (from B to F, according to Working Formulation) were suitable for minimal residual disease evaluation after HDS chemotherapy. Residual disease was assessed by polymerase chain reaction (PCR), using the bcl-2 oncogene or immunoglobulin heavy-chain (IgH) gene rearrangements as tumor cell markers. Rearranged variable regions (VDJ) have been amplified using one of two sets of sense primers (from the leader, and first framework region), and a consensus antisense primer derived from the 3' end of the six joining regions. Amplified VDJs have been directly sequenced or cloned, and the second and third complementarity determining regions (CDR2 and CDR3) identified. Tumor-specific oligonucleotides have been generated from CDR sequences. The use of a primer derived from the CDR2 and a probe from the CDR3, gave two steps of specificity to the residual disease detection: a tumor-specific amplification, and hybridization. In 20 of 24 patients (83%) a molecular marker was available (7 based on bcl-2 translocations, and 13 on IgH rearrangements). At present 45 PB and 15 BM cell harvests from 15 patients have been tested for the presence of residual lymphoma cells. In 4 of 15 patients (26%), lymphoma cells were not detectable in both PB and BM cell harvests. Among patients with PCR-negative cell harvests, 3 had a morphologically evident BM infiltration at diagnosis, and 1 was positive by PCR. Three main conclusions can be drawn from our data: i) the assessment of minimal residual disease by PCR is possible in most of B-cell lymphomas; ii) HDS chemotherapy can provide PCR-negative cell harvests only in a few patients; iii) the presence of residual lymphoma cells in most of the PB and BM cell harvests suggests that different therapeutic approaches such as the use of CD34+ cell purification and antibody-purged BM autografting need to be evaluated.

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PILOT STUDY USING HDS REGIMEN WITH INTENSIFIED DEBULKYING PRETREATMENT IN LOW/INTERMEDIATE GRADE NON-HODGKIN'S LYMPHOMA AT DIAGNOSIS

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The novel high-dose sequential (HDS) chemotherapy regimen, designed by the Milan Cancer Center, is based on the sequential administration of single drugs at their maximal tolerated doses, followed by a submyeloablative phase with autograft. The addition of hemopoietic growth factors along with the use of circulating progenitors allow safe delivery of the regimen. Indeed, a recent randomized study has shown good tolerability and potent antitumor efficacy of HDS in diffuse large cell non-Hodgkin's lymphoma (NHL) (*Gianni AM et al, Proc Am Soc Clin Oncol, Dallas 1994*). Based on these premises, we started a pilot study to evaluate feasibility and efficacy of HDS as upfront treatment for NHL other than diffuse large cell forms. So far, 21 patients aged 26-62 (median: 52) have been enrolled. Two patients had symptomatic follicular NHL (C subtype according to the W.F.); the remaining patients had intermediate grade NHL (7 E, 5 F, and 7 transformed subtypes). All patients presented with advanced stage disease and 13 had marrow involvement. The original HDS was employed in the initial 6 patients, while a more intensive debulkying pretreatment, including 2 APO and 2 DHAP, was introduced thereafter. There was one sudden death in a patient with previous acute endocarditis; 5 major infectious complications occurred and rapidly resolved following antibiotic therapy; no other severe treatment-related toxicities were recorded. One patient had disease progression, 3 patients did not go through the final phase due to either molecular (2 pts) or macroscopic residual marrow disease. CR was achieved in 15 patients (71%); 14 of them are in CCR at a median follow-up of 15 mos. In conclusion, HDS is feasible even following an intensified debulkying pretreatment; moreover, the results confirm the efficacy of HDS, as documented by the high CR rate in NHL subtypes usually showing a relatively low response to conventional chemotherapy.

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METHOTREXATE+AZT IN HIGH-GRADE HIV-RELATED NON-HODGKIN LYMPHOMAS

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3'azido 3'deoxythymidine (AZT) is clinically useful in the treatment of AIDS. We have previously demonstrated that this compound can possess a significant antineoplastic activity when combined with drugs that inhibit *de novo* thymidylate synthesis, such as 5-fluorouracil or methotrexate (MTX).

In this study we aimed to investigate the efficacy and the tolerance of the combination MTX+AZT in HIV-related non-Hodgkin lymphomas (NHL). 16 patients (10 males, 6 females) have so far been enrolled in the trial; the median age was 35 years, 8/16 had AIDS prior to lymphoma, 15/16 had less than 200 CD4/mmc.

Histological diagnoses were: Burkitt (5 cases), immunoblastic (2 cases), anaplastic large cell lymphoma (3 cases) centroblastic (4 cases), high-grade unclassifiable (2 cases); stage IV was present in 11/16 patients, with bone marrow involvement in 7 cases, hepatic involvement in 2 cases, CNS and pleural involvement in 1 case.

Patients received 3 consecutive courses of MTX 1 g/m^2 (days 1, 8 and 15) + oral AZT 2 g/m^2 (days 1, 2 and 3), 4 g/m^2 (days 8, 9 and 10) and 6 g/m^2 (days 15, 16 and 17). Folinic acid was administered 12 hours after each dose of MTX. From the 11th patient on, the treatment was continued with 3 more courses in case of complete or partial response. Out of 14 evaluable patients, 8 (57%) obtained complete remission and 4 (28%) showed a partial response. Median follow-up of the responder patients is 8 months (range 2-24). Grade III-IV neutropenia was observed in 35/59 courses. In no case was it observed extra-hematological toxicity. In conclusion, MTX+AZT appears to be effective and well tolerated in HIV-related NHL.

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RANDOMIZED MULTICENTER TRIAL WITH OR WITHOUT GRANULOCYTE COLONY-STIMULATING FACTOR AS ADJUNCT TO INDUCTION VNCOP-B TREATMENT OF ELDERLY HIGH-GRADE NON-HODGKIN'S LYMPHOMA

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Many elderly patients suffer from high-grade non-Hodgkin's lymphoma (HG-NHL); intensive chemotherapy is often complicated by severe bone marrow toxicity. This problem might be alleviated with granulocyte colony-stimulating factor (G-CSF). The effect of G-CSF on neutropenia, infection, and cytotoxic chemotherapy administration is ongoing in a randomized trial in patients receiving intensive weekly chemotherapy regimen. From march 1993 to may 1994 forty-nine patients with advanced stage HG-NHL (Kiel classification) with age >60 years have been treated with VNCOP-B regimen, a MACOP-B-like scheme with mitoxantrone 10 mg/m^2, cyclophosphamide 300 mg/m^2 on weeks 1, 3, 5, 7; vincristine 2 mg on weeks 2, 4, 6, 8; etoposide 150 mg/m^2 on weeks 2, 6; bleomycin 10 mg/m^2 on weeks 4, 8; prednisone 40 mg daily with dose tapering over the last 2 weeks. Twenty patients received VNCOP-B chemotherapy alone and 29 patients with G-CSF administration: 5 μg/kg/day troughout the treatment starting on day 3 of every week for 5 consecutive days. Neutropenia (absolute neutrophil count $<1.0\times10^9$/L) occurred in 5 of 29 (18%) of the G-CSF-treated patients and in 9 of 20 (45%) of the controls (P=0.03). The frequency of clinically relevant infections occurred in 2 of 29 (7%) of the G-CSF group and in 6 of 20 (30%) of the controls (P=0.03). The dose intensity of cytotoxic chemotherapy was increased in patients receiving G-CSF: median of 95% in G-CSF group compared with 88% in control patients. Complete response rates were 20 of 29 (69%) in G-CSF treated versus 11 of 20 (55%) in controls (P=0.31). These preliminary data show that VNCOP-B is a feasible and effective cycle in elderly HG-NHL patients and G-CSF significantly reduces infection and neutropenia; a larger number of patients is needed to confirm if the use of G-CSF may improve the response.

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FIRST RESULTS FROM A POPULATION CASE-CONTROL STUDY ON CHRONIC LYMPHOCYTIC LEUKEMIAS AND NON HODGKIN'S LYMPHOMAS ACCORDING TO HISTOLOGIC TYPE IN FARMING-ANIMAL BREEDING

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A population-based case-control study was conducted in a highly agricultural area in the North-East of Italy to evaluate the association between farming and animal breeding and the risk of developing non Hodgkin's lymphoma (NHL) and chronic lymphocytic leukemia (CLL).
Occupational histories and other data were collected by personal interview on 164 NHLs, 23 CLLs, diagnosed in 1988-90, and on 977 controls.
Within the *Farming/Animal breeding/Fishing working* category, two large sub-categories were identified: *farmer* (cultivation of land only) and *farmer-breeders* (also involved in animal breeding). Only the latter group presented a high risk for NHLs/CLLs (OR=1.79, 95% CI=1.22-2.63).
Farmer-breeders who stated personally using chemicals had an OR=1.99 (95% CI=1.28-3.08) for NHLs/CLLs compared to all other workers.
Analysis according to histologic type reveals that the risks are concentrated in CLLs and low-grade NHLs.

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LYMPHOMAS AND LEUKEMIAS: INCIDENCE DATA IN UU.SS.LL. 35-37-38 FOR YEARS 1986-91

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Since 1985 a population Registry has been operating in Romagna for studying cancer incidence. Hemolymphopoietic neoplasia are particularly frequent in our geographical area as they account for 8.4% and 8.7% of all tumors in males and females respectively. Between 1986 and 1991 the Romagna Cancer Registry recorded 380 leukemias, 513 non Hodgkin's lymphomas and 85 Hodgkin's lymphomas. These data refer to UU.SS.LL. 35-37-38 populations with an approximate total of 430,000 inhabitants.
Standardized (world) incidence rates per 100,000 persons were calculated for these hematological diseases for the years 1986-1991. In males lymphatic leukemias showed a higher incidence than myeloid leukemia (5.8 vs 4.2).
These data are due both to a higher number of acute lymphatic leukemia in male patients under 10 and a to a higher incidence of chronic lymphatic leukemias in patients over 50. In women we observed a higher incidence of acute myeloid leukemias (2.7) than in men (2.4).
This trend seems to be characteristic of our region and it may be a consequence of the higher number of cases that arise in females under 10.
Incidence rates of non-Hodgkin's lymphomas were 13.5 and 9.1 in males and females respectively.
These data show a higher nHL incidence in our region than that observed by other Cancer Registers. Among nHLs in men, in accordance with literature data, we observed an increased incidence in terms of percentage for extra-nodal patologies than for nodal ones, even though nodal pathology incidence is higher. The most commonly involved extranodal site was the stomach. In Hodgkin lymphomas, incidence was 3.5 for males and 3.1 for females.
Overall incidence was higher for patients under 45 years of age.

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EXPRESSION OF HEMATOPOIETIC GROWTH FACTOR AND OTHER CYTOKINE mRNAs DURING THE COURSE OF CHRONIC MYELOID LEUKEMIA

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Chronic myeloid leukemia (CML) is a lethal malignancy of the human hematopoietic stem cells characterized by the presence of the Philadelphia (Ph1) chromosome, which results from a molecular rearrangement involving the bcr and abl genes located on chromosome 22 and 9, respectively. This disease has two well-defined phases: the initial phase is characterized by a pronounced benign leukocytosis; the second by marked expansion of blast cells. During the first phase, despite the marked expansion of the malignant hemopoietic stem cells clone, normal hematopoietic stem cells may coexist with Ph1-positive stem cells. The reason for the proliferative advantage of the Ph1-positive stem cells is not known. We and others have shown that in other myeloproliferative diseases, such as acute myeloid leukemia the blast cells may autonomously produce hemopoietic growth factor and other cytokines and so establish an autocrine stimulation feedback of the proliferation.
The present study analyzes the expression of genes that encode various CSF's and some cytokines produced by myeloid cells in both the chronic phase and during blastic transformation in an attempt to ascertain whether the neoplastic myeloid population is able to produce cytokines autonomously; whether, if so, in the production is also involved the microenviroment, and whether an autocrine mechanism confers a proliferative advantage of the leukemic clone. The northern blot analysis and polymerase chain reaction technique were used to analyze the mRNA expression of the growth factors: interleukin 3 (IL-3), the granulocyte-colony-stimulating-factor (G-CSF), granulocyte-macrophage-colony-stimulating-factor (GM-CSF) and monocyte-colony-stimulating-factor (M-CSF) and of the cytokines: interleukin 1α (IL-1α) interleukin 1β (IL-1β), interleukin 4 (IL-4), interleukin 6 (IL-6) and leukemia inhibitory factor (LIF). Briefly the results were: when myeloid cells in various stages of differentiation during the chronic phases were analyzed together, they did not express mRNA for G-CSF, GM-CSF, M-CSF, IL-3, IL-4 or IL-1α. Whereas all cases examined expressed mRNA for IL-1β and IL-6, but only 58% cases expressed mRNA for LIF. The expression pattern differed during blastic transformation; 83% of cases expressed LIF mRNA while IL-1α, IL-1β, IL-6 mRNA were present in all cases. Moreover, although mRNA for GM-CSF and G-CSF were expressed during myeloid blast crisis, M-CSF was rarely seen. Such behaviour is reminiscent of the autocrine CSF and cytokine production pattern of ANLL blasts. Based on a comparison with normal CD34 positive cells cytokine production characteristics, we propose a self-sustaining model of the leukemic clone that is able to confer a proliferative advantage on the neoplastic clone through an autonomous stimulation mechanism.

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POSITIVE SELECTION OF CD34+ CELLS: A SHORT REVIEW OF THE METHODS CURRENTLY AVAILABLE FOR EXPERIMENTAL AND CLINICAL USE WITH PARTICULAR FOCUS ON IMMUNOMAGNETIC BEADS AND CHYMOPAPAIN

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In the rapidly expanding fields of stem cell transplantation and cell biology, the demand for purified or enriched populations of immature hematopoietic cells is increasing. Highly purified cells will facilitate studies of growth factors mediated stem/progenitor cell expansion and introduction of genetic material into stem/progenitor cells; at clinical level the selection of these populations reduces the load of contaminating malignant cells, and the volume of criopreserved and reinfused material can be decreased.
The so-called positive selection of stem/progenitor cells has been recently accomplished by sorting techniques that select these cells taking advantage of the expression of the CD34 antigen on their surface. Several immunoadsorption methods are presently available for research application, some of them being already applied *in vivo* in the human transplantation setting. They can be divided into 3 main groups: avidin-biotin immunoadsorption on column, panning, and immunomagnetic beads (with considerable variations among different manufactures in bead size, type of magnetic field applied, the antibody used, the use of direct or indirect separation techniques, and the detachment system).
Using small-scale devices for research purposes, CD34+ cells can be enriched from bone marrow, cord blood and mobolized peripheral blood to a purity ranging from 80 to 95% with differences between the systems concerning preparative steps, the recommendend maximal cell load, the actual processing time and the cell yield. To date only 3 systems have been applied to process whole grafts in large-scale devices (with purities and recoveries less brillant than using the corresponding small-scale system) and more than 200 transplants have been performed successfully.
In our laboratory we have developed the technique of immunomagnetic beads associated to the use of chymopapain to detach the cells from the beads. Sixteen samples (6 BM and 20 PB) from healthy volunteers or patients with CML and ANLL were studied. The mean percentage of CD34+ cells in the isolated fraction reached 85±10.6%, with a mean recovery of 64±22%. The method is very efficient even when initial percentage of CD34+ cells is very low (i.e. <5%)).

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CHARACTERIZATION AND SELECTION OF BENIGN STEM CELLS IN CHRONIC MYELOID LEUKEMIA

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Chronic myeloid leukemia (CML) is a clonal disorder arising from the hematopoietic stem cell, characterized by the Philadelphia chromosome (Ph) and, at the molecular level, by the fusion of the BCR (breakpoint cluster region) gene and the c-ABL gene. The hallmark of CML is represented by the marked increase of the number of leukemic progenitors, as well as more mature cells, in the bone marrow (BM) and peripheral blood (PB). Despite the expansion of the leukemic clone, normal Ph-negative stem cells have been demonstrated to survive in CML.
Early observations of partial, but transient, restoration of Ph- negative hematopoiesis after high-dose chemotherapy, have been recently extended by the use of myeloablative regimens followed by autografting with marrow or blood-derived stem cells. Moreover, treatment of early-stage CML patients with the biologic response modifier α-interferon (α-IFN), has led to the re-emergence of normal progenitor cells. Cuncurrently, *in vitro* studies have reported that cultures of CML marrow in the presence of a stromal feeder-layer resulted in the depletion of Ph- positive cells and the predominance of Ph-negative hematopoietic precursors. Based on the assumption that normal and malignant stem cells may coexist in CML, several studies have been recently directed to the characterization and *in vitro* selection of benign progenitors within CML hematopoiesis.
The results of those studies demonstrated that normal precursors can be phenotypically and functionally identified in the BM or PB of Ph-positive CML patients. These cells are included in the earliest identifiable hematopoietic cell compartment. Normal cells do not bear cell surface lymphoid or myeloid-lineage antigens, express high level of the CD34 antigen and fail to express the HLA-DR antigen. Furthermore, they possess a high capacity of adhering to the marrow stroma. This cell population represents only a small minority of hematopoietic progenitors but it retains many properties associated with the putative hematopoietic stem cells.

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CML: PH-NEGATIVE CELLS COLLECTED AFTER CHEMOTHERAPY CONTAINING LTC-ICS ARE ABLE TO RESTORE AND SUSTAIN POLYCLONAL HEMATOPOIESIS AFTER AUTOGRAFTING

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Thirty patients with CML in CP, 23/30 refractory to IFN, and 24 patient in AP have been treated with ICE (idarubicin+ARA-C+VP16) with the aim of collecting normal Ph-ve hematopoietic cells during the early phase of recovery. In 15 of CP and 6 of AP we have been able to collect aphereses containing Ph-ve methaphases only, whose cells were able to form $>1\times10^4$/kg GM-CFC and variable number of LTC-ICs (long-term culture initiating cells). Therefore patients fullfilling these criteria were, in principle, eligible for autografting. The remaining patients had either Ph+ve, Ph-ve mixed with Ph+ve collection. When we analyzed Ph-ve aphereses we found almost invariably presence of LTC-ICs (which were subsequently proven to originate from Ph-ve cells), even in absence of clonogenic cell growth: this pattern was observed mainly in the earliest aphereses. Overall LTC-ICs were quantitatively correlated with progenitor cells. The highest values of LTC-ICs and progenitor cells were collected from patients undergoing the procedure early after diagnosis; in these cases, progenitor Ph-ve cells were present also in the early aphereses; analysis of Ph+ve and mixed collections revealed either absence of LTC-ICs or presence of mixed LTC-ICs: in both cases progenitor cells were present and proved to be Ph+ve. In conclusion these data suggest: 1) Ph-ve cells can be recruited in peripheral blood in more than 50% of patients with CML in CP and only 25% of patients in AP: therefore the phase of the disease is the most important variable predicting the success of the procedure; 2) the collection seems more profitable in patients at diagnosis: this indicate that, in this stage, normal hemopoiesis is not enterely suppressed; 3) the cases of lack of progenitor cells in presence of LTC-ICs (the commonest pattern in patients with >1 year from diagnosis) suggests that normal hemopoiesis, after debulking, starts to prolifeate again from very primitive cells. This imply that normal hemopoiesis was completely inactive, and that this procedure can recruit into peripheral blood cells that were likely to be out of cycle; 4) Ph+ve collections generally do not give rise to LTC-ICs either because the exaustion of normal marrow either because of the rapid decline of leukemic cells in culture. Seventeen patients have been autografted with blood collected cells. Twelve were in CP, 5 in AP. Fifteen have been grafted with Ph-ve only, two with a mixture of Ph-ve and Ph+ve cells. Twelve are alive 2 to 29 months from autograft. Two patients died of transplant related mortality and three of progressive diesease. Two patients autografted with Ph-ve/Ph+ve cells reconstituted hematopoiesis with Ph+ve cells. Ten patients grafted with Ph-ve cells only, reconstituted Ph-ve hematopoiesis lasting from 2 to 29 months from transplant. Five of them relapsed (10 to 100% Ph+ve) 3 to 15 months after the procedure, and two of them are under interferon treatment. The other patients have sutained Ph-ve polyclonal hematopoiesis.

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MODULATION OF CELL KINETICS AND IN VITRO CELL GROWTH OF CML CD34+ PROGENITORS INDUCED BY p53 ANTISENSE OLIGONUCLEOTIDES

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Mutations of the p53 tumor suppressor gene occur in 20% of CML patients in blastic crisis, but it is still object of debate whether this inactivation may play a role in the pathogenesis of blastic transformation or in maintaining the leukemic proliferation in CML as it does in several solid tumors.
The aim of this study was to evaluate the effects of inhibiting p53 expression on proliferative activity of chronic phase CML (n.22) and normal (n.12) bone marrow CD34+ progenitor cells by using an 18-mer modified antisense oligonucleotide which target the region covering the 6 base pairs immediately before the first codon and the first 4 coding codons of p53. Modulation of cell kinetics has been assessed by estimating the percentage of positivity for the Ki67 antibody and by evaluating the CFU-GM production and the position into the cell cycle of CML and normal cells after their treatment with antisense, sense, and scrambled phosphorotioate oligonucleotide.
The results showed that treatment of CML cells with p53 antisense oligonucleotides abrogated p53 expression by either light density cells or purified CD34+ cells, accompanied by a significant increase in the number of cells positive for Ki67 and bromodeoxyuridine (BrdU) McAbs, and situated in active phases of the cell cycle (S,G2,M), as assessed by DNA analysis. Furthermore, the longer the incubation time, the higher the increase in cell proliferation.
Treatment of CML cells with p53 antisense oligomers also resulted in significantly increased numbers of CFU-GM colonies, thus confirming the inhibitory role played by wild type p53 on cell proliferation activity and *in vitro* cell growth.
We can speculate that the loss of p53 function at the time of blastic crisis of CML may play a role, together with other genetic changes (p210 BCR/ABL, Rb gene abnormalities, others- to be defined), in the transition from chronic phase to blastic crisis.

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AUTOLOGOUS BONE MARROW TRANSPLANTATION AFTER IN VITRO PURGING WITH BCR-ABL ANTISENSE OLIGODEOXYNUCLEOTIDES FOR PATIENTS WITH CHRONIC MYELOID LEUKEMIA IN ADVANCED PHASE

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Experimental and animal studies have indicated that BCR-ABL antisense oligodeoxynucleotides inhibit BCR-ABL expression in Ph-pos cells and that this inhibition may restore apoptosis in CML cells and prolong survival in *in vivo* treated leukemic mice. We have started a phase-1 trial of ABMT in patients with CML in advanced phase using bone marrow purged *in vitro* with 26-mer phosphorothioate BCR-ABL antisense oligonucleotides (Lynx Therapeutics INC, Hayward, CA, USA). Before BM harvest, progenitor cells were *in vitro* tested with oligonucleotides to evaluate the effective elimination of leukemic cells and to enumerate residual clonogenic cells after the *in vitro* treatment. Incubation with oligonucleotides was prolonged for 24 hours using a concentration of 150 ug/mL. Three patients, who showed at the *in vitro* test a significant inhibition of BCR-ABL rearranged clonogenic cells, were admitted to BM harvest. A total of 6.7, 8.0 and 5.4×10^9 mononuclear cells were respectively recovered after Ficoll separation containing 2.5, 43 and 4% CD34-positive cells and 1.0, 0.3 and 1.7×10^6 clonogenic cells, respectively. After the 24 hours incubation, 82, 84 and 48% mononuclear cells, 59, 88 and 84% CD34+ cells and 67, 84 and 74% clonogenic cells were recovered, respectively. One patient was autografted using the BCR-ABL antisense treated BM cells, after a conditioning regimen consisting of busulphan (16 mg/kg) and VP-16 (40 mg/kg). BM engraftment was observed at day +15 and platelets $> 50\times10^9$/L and neutrophils $> 0.5\times10^9$/L at day +18 and +25, respectively. The presence of non-rearranged cells was serially investigated from day +15 using both standard cytogenetics and fluorescence *in situ* hybridization (FISH). A greater sensitivity of FISH over the standard cytogenetics was demonstrated, showing a proportion of Ph-negative cells in repeated controls. The patient is in hematological complete remission 5 months after the autograft. Although preliminary, these results indicate that incubation with BCR-ABL antisense oligonucleotides does not affect pluripotent stem cells responsible for both short and long-term BM engraftment and that a proliferative advantage of the residual normal progenitor cells over the leukemic population might be obtained after the autograft.

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COLLECTION OF PRIMITIVE AND COMMITTED CLONOGENIC CELLS FOLLOWING MOBILIZATION OF CIRCULATING PROGENITORS WITH CHEMOTHERAPY PLUS G-CSF OR G-CSF ALONE

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High dose chemoradiotherapy associated with circulating progenitor cells (CPC) transplantation is increasingly used to treat hematological malignancies. Different mobilization regimens based on chemotherapy plus growth factors or growth factors alone have been proposed. The aim of this study was to quantitate the primitive and committed progenitor cells mobilized with chemotherapy and/or growth factor. Patients with multiple myeloma and non-Hodgkin lymphoma were included in this study. Six patients (Group 1) were mobilized with cyclophosphamide (7 g/m^2) and G-CSF (5 μg/kg/die s.c.) while seven patients (Group 2) were mobilized with G-CSF (10 μg/kg/die s.c.) alone. At the time of mobilization, all patients had been pretreated and had a variable percentage of bone marrow infiltration. CPC collections were performed when $\geq 20/\mu$L CD34+ cells (HPCA-2PE, Becton-Dickinson) were detected and were continued until $\geq 5\times10^6$ CD34+/kg cells were harvested. A continuous-flow blood cell separator was used. A median number of 3 apheresis (range 2-4) in Group 1 and 2 apheresis (range 1-3) in Group 2 were performed. The mean values of primitive and committed progenitors in the collected material are reported below.

	group 1	group 2	p
nucleated cells/kg	3.62×10^8	7.06×10^8	<.05
CD34 cells/kg	6.41×10^6	6.94×10^6	ns
week 5 CFU/kg	6.85×10^5	1.20×10^4	<.05
CFU-Mix/kg	1.38×10^4	1.48×10^4	ns
BFU-E/kg	1.84×10^5	1.74×10^5	ns
CFU-GM/kg	1.05×10^6	2.83×10^5	<.05

In three MM patients undergoing CPC mobilization with G-CSF alone, CD34+ cells were selected by avidin-biotin columns (CEPRATE® SC Stem Cell Concentrator). Positive selection resulted in a 63-fold enrichment of CD34+ cells. A mean value of 3.22×10^6 cells/kg were cryopreserved. Clonogenic cell recovery and enrichment were 51% and 117-fold, respectively. Interestingly, a 2 to 5 log reduction of CD19+ B cells was achieved by this procedure. In conclusion our data show: (1) the possibility to collect CPC in pretreated patients with marrow involvement; (2) the ability of G-CSF to augment nucleated and CD34+ circulating cells without chemotherapy administration; (3) a significantly higher content of primitive progenitors after mobilization with chemotherapy plus G-CSF as compared to G-CSF alone; (4) the feasibility of enriching CD34+ cells for clinical use by CEPRATE® SC Concentrator; (5) a significant reduction of CD19+ cells following CD34 enrichment.

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MOBILIZATION OF CIRCULATING HEMATOPOIETIC PROGENITOR CELLS (CHPC) BY STANDARD-DOSE CHEMOTHERAPY + G-CSF IN BREAST CANCER PATIENTS

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Introduction. High-dose chemotherapy plus hematopoietic growth factors is a commonly utilized method to mobilize circulating hematopoietic progenitor cells (CHPC). However, it requires hospitalization and is not without toxicity. Present study was performed to evaluate the possibility to mobilize CHPC by a standard-dose chemotherapy plus granulocyte-colony stimulating factor (G-CSF).
Methods. 11 patients with breast cancer were treated with 6 cycles of CEF14d (cyclophosphamide 600 mg/m^2, epirubicin 60 mg/m^2, fluorouracil 600 mg/m^2 i.v., day (d) 0, q.+14 d) + G-CSF 5 μg/kg/day s.c. from d 3 to d 10. On d 0 and from d 3 to d 14 throughout the first 3 cycles peripheral blood samples were collected and evaluated for the content of CHPC, determined as the number of CFU-GM/mL, BFU-E/mL, CFU-Meg/mL as well as the number of CD 34+ cells/μL.
Results. At baseline, as expected, low mean values (range) of CHPC were observed: CFU-GM 46 (0-144), BFU-E 229 (16-431), CFU-Meg 10 (0-23). Similarly, low numbers of CD34+ cells were observed 3 (0-7) before chemotherapy. After CEF and G-CSF therapy a relevant increase was observed in both circulating CFU-GM and CD 34+ cells in all patients, always after d 9. Mean peak values at the 1st, 2nd, 3rd cycle of both CFU-GM and CD34+ cells were observed between d 10 and d 11 and were 1187 (±331), 890 (±277) and 513 (±203), respectively, for CFU-GM, and 193 (±81), 143 (±53) and 123 (±81), respectively, for CD34+ cells. A positive correlation, estimated by linear regression analysis, was observed between CFU-GM and CD34+ cells (r=0.69). In all patients this treatment regimen was well tolerated and almost without hematological toxicity.
Conclusions. Our data indicate that CHPC can effectively be mobilized by standard-dose CEF chemotherapy + G-CSF without any relevant toxicity. These CHPC could be collected by some leukaphereses performed after d 9 in order to support further high-dose chemotherapy.

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PERIPHERAL BLOOD STEM CELL AUTOTRANSPLANT FOLLOWED BY GM-CSF: IMMUNOLOGICAL PROFILE

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The immunological profile after autotransplant of peripheral blood stem cell (PBSC) followed by GM-CSF was evaluated by measuring serum levels of IL-2 and CD8 soluble receptors; IFN-γ, IL-2, IL-6, IL-1α, IL-1β and neopterin (Np) in patients affected by multiple myeloma (MM), Hodgkin disease (HD) or low-grade non-Hodgkin lymphoma (LG-NHL) before (day –7 and 0) and after (every 5 days from +5 to +40 and day +60) transplantation. Intracellular levels of all cytokines were studied on days –7 and 0, day +15 and then as for serum concentrations. Percent and absolute numbers of cells that expressed a T, B or NK phenotype, and NK function, were determined on days –7, +20 and +40. Serum levels of the IL-2 receptor increased 3-fold over basal values from days +5 to +15, then tended to drop, but returned to pre-transplant levels only on day +60. Serum levels of CD8 soluble receptor rose from day +15 and reached maximum values of 3.5-fold over pre-transplant values from days +25 to +35. Although they then gradually reduced, they were still high on day +60. There were no significant variations in IFN-γ, IL-2 or IL-6 serum levels. Intracellular levels also remained unchanged, but the small number of cases studied does not allow conclusions to be drawn. In contrast, despite wide variation between individual patients at all study times, serum values of IL-1α and IL-1β displayed a marked tendency to increase post-transplantation. IL-1α concentrations reached values 3.5, and IL-1β 2.5, times greater than basal levels from days +30 to +60. Intracellular concentrations of IL-1β followed a similar trend, but with wide interindividual variations, whereas IL-1α values remained unmodified. Serum Np also rose, it was twice the basal value on day +10 and 4 times higher from days +15 to +40. Natural killer cell activity increased in the 7 patients with initially low values, but not in the 3 whose basal values were normal. Percent values of cells that express T markers were reduced on day +20, but were superimposable on basal value by day +40 except for an expansion in the CD8+ pool. There were no noteworthy post-transplant percent variations in other lymphocyte populations.

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HIGHLY EFFICIENT PURIFICATION OF CD34+ HEMATOPOIETIC PROGENITOR CELLS BY HIGH-GRADIENT MAGNETIC CELL SORTING

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Introduction. Most of the methods at present available for isolation of CD34+ hemopoietic progenitor cells (HPC) utilize large surfaces of binding, using either direct or indirect immunospecific labelling techniques. However, these systems often have a consistent rate of unspecific cell binding due to large surface of immunoaffinity, and potentially reduce vitality of purified HPC. Recently a new separation technique (MACS Systems Miltenyi Biotec Gmbh, Bergisch, Germany) has been proposed, which utilizes a very smaller surface-binding minimizing unspecific binding and interference with successive manipulation. Aim of our work was to verify the efficiency and reliability of this method of isolation for successive *in vitro* expansion of CD34+ HPC.
Methods. Peripheral blood mononuclear cells (PBMC), harvested by apheresis following high-dose therapy plus GM-CSF and further purified by Ficoll gradient centrifugation, were resuspended in PBS-5mM-EDTA-0.5% BSA at the concentration of 3.5×10^8 cells. PBMC were then incubated for 15' with an anti-CD34 antibody (Ab) (QBEND/10-100 μL/10^8 cells). Colloidal superparamagnetic microbeads recognizing CD34 Ab were subsequently added and PBMC were further incubated for 15' at 6°C. PBMC were resuspected again in PBS-5mM-EDTA-0.5% BSA and flushed through a separation column adherent to a magnetic support for positive selection of CD34+ cells.
Results. In five consecutive experiments the percentage (mean) of CD34+ cells before magnetic cell sorting, as determined by FACS analysis using a different anti-CD34 Ab (HPCA-2), was 10.9% (range 2.6%-19.9%). After high-gradient magnetic cell sorting, the mean percentage of CD34+ cells was 93.7% (range 80.1%-98%). The percentage of CD34+ cell recovery was 43% (range 10.6%-59.4%). These sorted CD 34+ cells appared be viable and capable of subsequent *in vitro* growth.
Conclusions. Using present technique we have obtained a reliable, time-saving and efficient isolation of CD34+ HPC, which allowed us for the availability of a consistent number of highy purified CD34+ HPC for further *in vitro* manipulations.

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EFFECT OF BONE MARROW STROMAL CELLS ON OSTEOCYTIC CELLS LINES

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Stromal cells in the bone marrow provide the cytokines, extracellular matrix and direct cell-cell interactions required for the maturation and proliferation of lymphoid and myeloid cells. Other cell types such as osteocytes, adipocytes and chondrocytes are present and may be relevant in determining the bone marrow microenvironment. We therefore analyzed the relationships between bone marrow stromal cells (BMSC) and osteocytic cells *in vitro*.
Primary cultures of BMSC were obtained as previously described (*Degrassi et al., PNAS USA 1993, 90:2060*) and three human osteosarcoma cell lines HOS, MG-63 and U-2 OS were obtained from ATCC (American Type Culture Collection). 3H-Thymidine incorporation of the osteocytic cells was evaluated 24 and 48 hours after co-culture with irradiated BMSC or after incubation with supernatants from either resting or γ IFN + TNFα activated BMSC.
The proliferation of MG-63 and U-2 OS cells was significantly inhibited (40%) when cells were co-cultured with BMSC while the proliferation of HOS cells was not affected suggesting that the growth inhibitory effect was not due to degradation of the medium. Furthermore similar results were obtained incubating the osteocytic cells with supernatants from BMSC. Activation of BMSC with γ IFN + TNFα did not affect the proliferative inhibitory effect of BMSC.
These preliminary data suggest that BMSC produce soluble factor(s) that can modulate the proliferation of osteocytic cell lines *in vitro*.
Future experiments will therefore be important to identify at the molecular level the relationships between BMSC and the osteocytic cells in the bone marrow microenvironment.

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IN VITRO GROWTH FRACTION EVALUATION OF LYMPHOID BLAST CELLS FOLLOWING EXPOSITION TO GM-CSF

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In order to evaluate the *in vitro* effect of GM-CSF on the proliferation of lymphoid blast cells we performed a short term liquide culture study employing leukemic cells from 16 patients with acute lymphoblastic leukemia at diagnosis or at relapse. Leukemic cells were obtained from bone marrow samples by Ficoll separation and cultured in RPMI with 10% FCS. Cell proliferation was measured by DNA content (S-phase) and 3H-thymidine (3H-thy) incorporation after 24 hour exposition to GM-CSF (Schering-Plough) at two different concentrations (10 and 100 ng/mL). For the DNA content method, cells were seeded at a concentration of 3×10^6/mL without (negative control) and with GM-CSF. DNA analysis was performed by FACS following propidium iodide staining. As far as 3H-thy incorporation is concerned, cells were incubated in a 96 microwell plate at a concentration of 2×10^5/mL without and with GM-CSF; tests were done in triplicate. After 16 hour incubation, cells were exposed to a 8 hour pulse with 3H-thy (0.5 Ci/well) and then were harvested; 3H-thy incorporation was measured by a counter. K562 cell line and blast cells from AML patients were used as positive controls.
Our results point out that:
a) DNA content and 3H-thy incorporation are equivalent and comparable methods to study cell proliferation;
b) GM-CSF does not cause any increase of the growth fraction of leukemic lymphoid cells;
c) in some cases GM-CSF at the highest concentration seemed to reduce cell proliferation, perhaps via apoptosis.
These studies suggest that GM-CSF could be safely administered to patients suffering from lymphoid neoplasm.

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PLASMA SOLUBLE STEM CELL FACTOR LEVELS IN PATIENTS WITH ACUTE NON-LYMPHATIC LEUKEMIA

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Stem cell factor (SCF) is a newly identified growth factor produced by bone marrow (BM) stromal cells and active in the early stages of hematopoiesis. Human SCF can exist in membrane-bound form and in proteolitically released soluble form, both with biological functional activity.
Soluble SCF is present in human serum at levels readily detectable by immunoassay and a reduction of soluble SCF has been found in patients with *aplastic anemia* (*Wodnar-Filipowicz A., et al., Blood, 81:3259-64, 1993*).
To obtain more information on the pathophysiology of *acute non lymphatic leukemia* (ANLL), we measured the concentration of soluble SCF in peripheral blood plasma of 18 patients with the disease and 15 healthy controls. In 17 patients SCF levels were also measured in BM plasma collected on the same day as peripheral blood. BM and peripheral blood were collected into EDTA and plasma SCF levels determined using a commercially available enzyme-linked immunosorbent assay (Amersham International plc, Biotrak). ANLL (at diagnosis, in relapse, RAEB-t in evolution) was classified according to the FAB criteria: 10 patients had AML M1, 4 AML M2, 2 AML M4, 1 AML M5 and 1 AML M6.
Soluble SCF levels in peripheral blood plasma of the patients were markedly lower than those of the controls (1055.1±298,45 vs 1626.9±297.79 pg/mL). Moreover, SCF in peripheral blood plasma correlated well with that in BM plasma in all the patients studied (R=0,91), indicating that the former reflects SCF production in BM stroma. In addition, a short follow-up of 3 patients during therapy showed an increase of SCF plasma concentration to normal values in the remission phase.
The decrease of soluble SCF found in patients with ANLL may depend on an inadequate supply of the factor in this hematopoietic disorder. As evidence suggests that membrane-bound SCF has functional importance, further studies on the biological activity of this cytochine are required to assess its role in the pathophysiology of ANLL.

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DETERMINATION OF ERYTHROPOIETIN LEVELS AFTER PERIPHERAL BLOOD PROGENITOR CELL TRANSPLANTATION (PBPCT): COMPARISON WITH AUTOLOGOUS BONE MARROW TRANSPLANTATION

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Erythropoietin (Epo) levels were evaluated by an immunoenzymatic method in 10 patients affected by hematological malignancies submitted to PBPCT (group A) after conditioning regimen BuCy2 and in 5 patients submitted to autologous BMT (group B) using the same conditioning regimen.
No correlation was found between Epo and hemoglobin levels in group A, while a strong negative correlation was observed in group B (r=-0.61, p=0.025). Comparing the two groups (group A 218 mU/mL, group B 417 mU/mL) a highly significant difference was observed (p=0.001). The different behaviour of Epo in the two groups reflects both the degree of anemia and the rapidity of hemopoietic recovery. This is also testified by the lack of transfusion requirement and by the early appearance of reticulocytes in group A compared to group B.
However, our data confirm that Epo levels post-autologous BMT are appropriate and there is no need for exogenous Epo administration. According to our experience, the same consideration should also be extended to PBPCT setting.

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INTERLEUKIN 1 AND GRANULOCYTE MACROPHAGE COLONY STIMULATING FACTOR REDUCE THE IN VITRO INHIBITORY EFFECT OF AN AZIDOTHYMIDINE INTERFERON α COMBINATION ON HUMAN HEMOPOIETIC PROGENITORS

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Both azidothymidine (AZT) and interferon-α (IFN-α) have an *in vitro* and *in vivo* antiretroviral activity against the immunodeficiency virus type I. The mechanisms of antiretroviral activity of the two drugs are distinct, AZT acting on the earlier and IFN-α on the later stages of HIV replication. Preclinical and clinical studies with an AZT, IFN-α combination resulted in a synergistic antiretroviral activity. A marked myelosuppressive effect was, however, observed and in fact each of both drugs directly inhibits the earlier stages of hemopoietic progenitor cells. *In vitro*, interleukin 1 (IL-1) and granulocyte macrophage colony stimulating factor (GM-CSF) have shown significant activity in modifying hemotoxicity of AZT. The *in vitro* activity of cytokines on hemotoxicity of the AZT, IFN-α association have never been studied. In this *in vitro* study we evaluated the toxicity of the AZT, IFN-α association on peripheral blood human hemopoietic progenitor cells (granulocyte-macrophage colony forming units, CFU-GM and erythroid burst forming units, BFU-E) and the activity of IL-1, GM-CSF or both the cytokines in modifying the AZT, IFN-α hemotoxicity. Results indicate that AZT, IFN-α and combinations of the two drugs have a dose-dependent inhibitory effect on *in vitro* growth of peripheral blood hemopoietic progenitors. Combinations of AZT and IFN-α inhibited the CFU-GM and BFU-E proliferation in an additive manner. Neither IL-1 nor GM-CSF separately were able to induce a significant reduction of AZT or IFN-α induced damage. Only the addition to the cultures of both cytokines partially countered the antiproliferative activity of AZT at low dosages, alone or in combination with IFN-α. This effect was particularly evident in the case of CFU-GM growth. These data suggest a possible role of an IL-1, GM-CSF association in attempts to improve the therapeutic index of the combination AZT, IFN-α in patients with AIDS.

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IL-6 SERUM LEVELS IN PATIENTS AFFECTED BY HEMATOLOGIC MALIGNANCIES: CORRELATION WITH NEUTROPENIA AND INFECTIONS

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Interleukin-6 (IL-6) is a multifunctional cytokine that plays a central role in the defence mechanisms including acute phase reactions, immune response and hematopoiesis.
In our study we have measured the levels of IL-6 in 11 patients (mean age 39.5; range 17-69) affected by hematologic malignancies (7 ANLL, 3 ALL, 1 NHD).
We collected serum samples from these patients every two days during the period of hospitalization when they were treated with antiblastic chemioterapy and suffered from severe neutropenia (absolute neutrophil count $< 0.5\times10^9$/L).
Every week all patients underwent a complete microbiologic test battery (nasal, rettal and oral swabs, urine colture, serum determination of Candida and Aspergillus Ab and Ag, CMV, HSV, HZV, EBV, HBV, HCV Ab); blood coltures were collected in the case of fever (temperature higher than 38°C).
The serum samples were centrifuged and stored at –30°C within 2 hours from the collection.
IL-6 levels were dosed by a radioimmunoassay (Advanced Magnetic In). Totally we collected 73 samples: 41 during febrile attacks, 27 in afebrile periods and 5 in correspondence of a septic shock state.
Twenty samples from normal donors were tested as controls.
In febrile patients IL-6 levels (mean 54.6±17.3 pg/0.1 mL) were elevated in comparison either with afebrile neutropenic patients (mean 41.0±37.9 pg/0.1 mL) (p=0.01) or with controls (mean 6.9±2.5 pg/0.1 mL) (p=0.0001).
IL-6 elevation showed only a weak correlation with fever (r2=0.094; F-test: p=0.01).
Interestingly the highest values of IL-6 (more than two hundred-folds up the normal range) have been found in patients suffering from septic shock.
IL-6 appears to be involved in the inflammatory response to a septic injury. The correlation found between IL-6 levels and the severity of the infective episodes appears to assign a negative prognostic value to IL-6 elevation and opens theoretically new perspectives in monitoring severely ill patients at risk of infection.

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IN VITRO EFFECTS OF rhSCF ON MEGAKARYOCYTIC COLONIES IN MDS PATIENTS

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The clonogenic growth of bone marrow (BM) cells from patients (pts) with myelodysplasic (MDS) is reduced *in vitro* even in the presence of high doses of growth factors such as EPO, GM-CSF and IL-3. Although the *in vitro* stimulatory effect of rhSCF on the erythroid and myeloid colonies of the BM cells of MDS pts is well known, *in vitro* megakaryocytopoiesis has been little studied. We therefore evaluated the megakaryocytic colony formig capacity of BM cells from 25 MDS pts (4 RA, 4 RARS, 11 RAEB, 3 RAEB-t and 3 CMML). The mononuclear cells were separated using gradient centrifugation and then subjected to one cycle of adherence to plastic flasks. Colony assay: CFU-MK and BFU-MK: 3×10^5 cells/mL were cultured in plasma clots with rhGM-CSF (200 U/mL) and rhIL-3 (100 U/mL), with and without rhSCF (2 U/mL). In comparison with normal controls, megakaryocytopoiesis in MDS was greatly reduced by the addition of rhIL-3 and rhGM-CSF: CFU-MK and BFU-MK growth was observed only in 33% and 31% of cases respectively. *In vitro* growth and response to rh SCF was quite variable. RhSCF stimulated CFU-MK in 47% of cases (MDS: $p<0.01$) and BFU-MK in 36% of cases (MDS: $p<0.05$); the number of colonies reached that of our normal controls without rhSCF particularly in RA. The increase in RARS and RAEB was less than that observed in RA; no growth was observed in RAEB-t and CMML. No linear correlation was found with the number of platelets in pheripheral blood.
These data suggest that rhSCF is capable of reversing defective megakaryocytopoiesis in MDS pts at low risk and that, with their progression to high risk, the progenitor cells become poorly responsive to rhGM-CSF, rhIL-3 and rhSCF stimulation. The same was confirmed for erythropoiesis and myelopoiesis. RhSCF may play a therapeutic role in megakaryocytopoiesis in a subset of MDS pts.

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LONG-LASTING HEMATOLOGICAL REMISSION OF REFRACTORY ANEMIA AFTER A SHORT COURSE OF GRANULOCYTE-MACROFAGE COLONY STIMULATING FACTOR (GM-CSF): A CASE REPORT

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Treatment of myelodisplastic syndromes (MDS) is very controversial and generally disappointing, 20 to 30% of patients have been reported to respond to recombinant human eriythropoietin (rh EPO). *In vitro* studies in MDS have shown maturational disturbancies at the level of the earliest committed erythropoietic stem cell (BFU-E), a cell that is less responsive to EPO than CFU-E, while requiring the presence of multilineage growth factors for its maturation. *In vivo* trials employing interleukin 3 have generally failed, while some result has been accomplished using GM-CSF.
Here we report a case of a long-lasting remission after a short course of GM-CSF.
S.A., a sixty-four year old female, was diagnosed as bearing a refractory anemia in September, 1986. After three years of follow-up she was admitted to our Institution for severe anemia (Hb 55 g/L) that required the transfusion of 6 to 8 packed red cells/months.
A course of GM-CSF was started at the dose of 5 μg/kg BW daily, subcutaneously. After ten days, such a treatment was discontinued due to leukocytosis (59.8×10^9/L WBC). The patient was then maintained on an ambulatory transfusion regimen, but soon after the discharge the transfusional requirement pregressively decreased. In March 1991, 13 months after the GM-CSF course, any transfusion was stopped. At the present, after more than 3 years of follow-up, she is well, with Hb levels permanently greater than 115 g/L.
Treatment with GM-CSF in MDS has to be kept in mind as a different opportunity to treat these syndromes.

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THE GROWTH INHIBITION OF HUMAN LEUKEMIC BLASTS BY QUERCETIN INVOLVES THE INDUCTION OF TRANSFORMING GROWTH FACTOR β

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Previously, we have demonstrated that quercetin (3,3',4',5,7 pentahydroxyflavone) inhibits in a dose dependent manner the growth of acute leukemias and it is able to enhance the antiproliferative activity of ARA-C. Here we demostrated that the induction of transforming growth factor β1 (TGF-β1) in leukemic blasts is one of the growth inhibitory mechanisms of quercetin in these cells.
We studied by clonogenic assay the growth inhibitory action of quercetin in 24 acute leukemias (AL) (4 M1-AML, 3 M2-AML, 3 M3-AML, 4 M4-AML, 3 M5-AML and 7 ALL) and we found that all but 2 were sensitive to this compound. In sensitive cases antisense TGF-β1 oligonucleotide at 8 μM concentration prevented the growth inhibitory action of quercetin.
Moreover we found that anti-TGF-β1 neutralizing antibodies were able to prevent almost completely the growth inhibitory activity of quercetin. Finally quercetin-sensitive cases when treated with this compound secrete in the medium large amount of TGF-β1 and show cellular TGF-β content when stained with a specific antibody against human TGF-β molecule.
In a quercetin-resistant case (M2-AML) TGF-β1 antisense alone produced more than 50% cell growth inhibition and anti-TGF-β1 antibody inhibited almost completely leukemic cell growth. In the other quercetin-resistant case (M1-AML) TGF-β1 antisense did not produce any effect.
In conclusion, quercetin can act as cytostatic agent for leukemic cells by modulating the production of TGF-β1. In rare quercetin-resistant cases TGF-β1 is either ineffective or stimulatory on leukemic cell proliferation.

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INDUCTION OF FETAL HEMOGLOBIN BY BUTYRATE ANALOGUES IN ERYTHROID LIQUID CULTURE FROM HUMAN PERIPHERAL BLOOD STEM CELLS

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Incraesed production or prolonged expression of fetal hemoglobin (HbF) can ameliorate the clinical course of thalassemia syndromes. Several chemoterapeutic agents have been shown to stimulate HbF, however because of the long-term nature of the treatment, concern has been raised about the toxicity and the potential carcinogenicity of these drugs. Butyric acid, a natural fatty acid, has been recently shown to stimulate HbF production *in vitro* and *in vivo*. The current method so far required for butyrate administration (long-term continuous intravenous infusion) is impractical for broad application in clinical trials and it raises an urgent need for other members of this class of compounds orally administrable.We report here the effect of butyric acid, sodium phenylbutyrate(NaPB) and sodium phenylacetate(NaPA) on cell differentiation, proliferation and hemoglobin F induction in a liquid culture system derived from normal human peripheral blood stem cells.The liquid culture procedure was set-up according to Fibach with minor modifications and consisted of two phases.Differentiation was assessed morphologically on cytocentrifuged slides and by flow cytometric analysis of the cell membrane antigens using different monoclonal antibodies (MoAb Immunotech). The Hb-containing cells were detected by benzidine staining. The total Hb level in lysates prepared from cells harvested at different days of culture was determined by HPLC (Bio-Rad). Beta and gamma globin mRNA prepared from control cells and from cells exposed to the drug effect in culture, were detected by a sensitive S1 Mapping. Different experiments were performed to evaluate the dose-response of erythroid precursors. Drugs have been added at different days of culture.The treatment of cultures by drugs resulted in cell proliferation reduction that was markedly evident as early as the drugs were added. The butyric acid effect was evident at 0.5-1 mM whereas NaPB and NaPA inhibited cell proliferation at higher concentration (2.5-5mM). The analysis of surface antigens such as CD34, CD36, glycophorin and transferrin receptor documented a reduction of cell differentiation following drug treatment. Beta globin mRNA was detectable in cells harvested from untreated cultures after 12 days (7 days phase 2) while gamma globin mRNA was faintly detectable after 15 days. Increased levels of gamma-globin mRNA in cells treated were shown in relation to the concentration of the drugs studied. The fold increase of mRNA is time-dependent being more evident when drugs were added in cultures when the cell population was mainly represented by erythroid precursors (days 10 phase 2)..

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RECOMBINANT ERYTROPOIETIN FOR THE TREATMENT OF ANEMIA IN SELECTED PATIENTS WITH MYELODISPLASTIC SYNDROMES.

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Myelodysplastic syndromes (MDS) are clonal disorders of the pluripotential stem cell characterized by peripheral blood cytopenias in the presence of a selective or multiple bone marrow hyperplasia/dysplasia. The main causes of death in MDS are infections, hemorrage and, depending on the subtype, trasformation to acute myeloid leukemias (AML); however, anemia is often the most important clinical problem degrading the quality of life in these patients. No effective treatment for anemia in MDS is currently available, the only exception being the rare patients in whom allogeneic bone marrow transplantation is feasible. Thus, supportive care with red cell transfusion, with its well-known risks related, remains the therapeutic approach for such patients. Recently, recombinant DNA technology has enabled to develop erythropoietin (rhEPO) as a therapeutic agent, and same published studies have already assessed the clinical effects of its administration in the treatment of anemia in MDS.
In this study, we used rhEPO to treat 10 consenting patients affected by MDS, whose major complaints were related to anemia. No severe hepatic, renal or hemostatic dysfunction were present. None of patients had received chemotherapy or other drugs aimed at correcting the bone marrow functional alterations (androgens, vitamins, steroids, retinoids, low-dose aracytin) for at least a month prior to the beginning of rhEPO. According to the French-American-British (FAB) criteria, three patients were classified as refractory anemia (RA), two as refractory anemia with ringed sideroblasts (RAS) and five as refractory anemia with excess of blasts (RAEB). rhEPO was given subcutaneously at a daily dose of 100 U/Kg for three months. A stable, non transfusion-supported, increase of 1 gr/dL in Hb concentration was considered as a complete response (CR), and a reduction of 50% in red cell transfusions as a partial response (PR).
A dose of 100 U/Kg/day induced an elevation of Hb levels in 5 of 10 patients (2 CR, 3 RP). In one case trasfusions were reduced although not enough to halve the number of red cells units per month. After an additional 3 month of treatment, evaluation of the 5 responding patients revealed that one had a progressive rise in Hb level and achieved a complete response, 2 showed no futher improvement of transfusional requirement, while Hb levels were immodified in the other 3 patients. The treatment was well tolerated in all patients and no adverse reactions were observed.
Thus rhEPO is a safe but not resolutive drug for MDS; nevertheless, some of these patients may benefit from this therapy. Our data seem more optimistic about the efficacy of rhEPO in correcting anemia in MDS than those published by other authors; in fact 50% of our patients showed a clinical response to standard dose of the drug. Further studies will be necessary to delineate the characteristics of patients who are likely to respond to rhEPO and to better define the range of dose required for a successful treatment.

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SERUM ERYTHROPOIETIN INCREASE IN ABSENCE OF ANEMIA FOLLOWS ADJUVANT THERAPY WITH 5FU-LV

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Experimental and clinical studies show a rise of serum EPO (sEPO) level, not related to the degree of anemia, in a setting of reduced erythropoietic proliferation following intensive cytotoxic chemotherapy, thus introducing the possibility that factors different from tissue hypoxia act on EPO regulation. Purpose of this study is the evaluation of sEPO changes following administration of a single anticancer drug (leucovorin modulated 5-fluorouracil; 5FU-LV) in a setting of adjuvant therapy following surgery for colon cancer. The study was performed on 7 patients, three males, four females, affected by colon cancer, pathological stage Dukes C2. Four weeks following cancer removal, the patients, all with a Karnofsky index of 100%, steady hematological data and a normal iron status, received adjuvant chemotherapy based on administration of LV 100 mg/mq i.v. and 5FU 300 mg/mq i.v. for 5 days. During the first course of therapy on day 5, 15, 28, the sEPO level, the peripheral blood (p.b.) reticulocyte level and Hemoglobin (Hb) concentration were assayed. Results show steady concentration of Hb, sharp decrease of p.b. reticulocytes and an increase of sEPO level which at day 15 following the start of 5FU-LV administration is two-fold the initial value. The maximum increase of sEPO level follows by ten days the nadir of p.b. reticulocyte level whose marked reduction reflects eryhropoiesis depression by the cytostatic drug. The increase of sEPO concentration observed in this study is much less striking than that detected after heavy doses of cytostatics but is however significative. The increase of sEPO concentration not triggered by anemia, but following cytostatic treatment has not yet a definite explanation but increased EPO synthesis remains the most acceptable possibility. It may be due to a direct stimulating effect of chemicals on renal oxygen sensors or on extra-renal EPO production. Finally, an unknown stimulus triggered by bone marrow inhibition might act on the kidney.

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REGULATORY ACTION OF PROLACTIN ON THE IN VITRO GROWTH OF CD34+VE HUMAN HEMOPOIETIC PROGENITOR CELLS

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The pituitary hormone *prolactin* (Prl) has been proposed to play a regulatory role on the immune cell function and Prl-binding sites (Prl-R) have been described to share distinctive features in common with the members of the hemopoietic growth factor receptor superfamily. Here we show that the hormone can directly modulate the *in vitro* growth and differentiation of normal hemopoietic progenitor cells (CD34+ve) induced by specific hemopoietic factors.
Enhanced number of colony forming unit-granulocyte (CFU-G) and burst forming unit-erythroid (BFU-E) colonies was observed in the presence of physiological to supra-physiological doses of Prl.
Enhanced proliferative response was found when CD34+ve cells were stimulated in liquid culture with granulocyte-macrophage colony stimulating factor (GM-CSF)/interleukin (IL)-3 in combination with Prl. In addition, pre-incubation with Prl increased the erythropoietin (Epo)-responsiveness of GM-CSF/IL-3 primed BFU-E by inducing an enhanced membrane expression of Epo-R. Analysis by flow cytometry using human cross-reacting PrR-7A monoclonal antibody (mAb), raised to the rabbit Prl-R, indicated that CD34+ve progenitors express PrR-7A molecule. Immunoprecipitation and biochemical studies of this antigen from the membrane of metabolically labelled CD34+ve cells showed a 43 kD single-chain structure similar to that expressed by the breast carcinoma cells T-47D.
These data suggest a possible receptor-mediated regulatory action of the hormone on normal hematopoiesis.

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EFFECT OF PROCAINE HYDROCHLORIDE ON MYELOTOXICITY INDUCED BY CARBOPLATIN IN NORMAL MICE

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Previous researches have shown in mice bearing P388 leukemia that the membrane active local anesthetic procaine hydrochloride (PHCl) is able to reduce the renal and to a lesser degree the hematological toxicity of cisplatin without interfering with its antitumor activity. We have performed an experimental study to evaluate if PHCl is able to induce protection from the myelotoxicity of carboplatin (CBDCA). The latter is a second generation platinum coordination complex, active as antineoplastic agent whose clinical usefulness is limited by the occurrence of severe myelotoxicity.
We have studied at fixed times the effect on the peripheral blood leukocytes, reticulocytes and pluripotent (CFU-S) and committed (GM-CFU, BFUe, CFUmeg) hemopoietic progenitors of a single i.v. injection of CBDCA (120 mg/kg), administered alone or in combination with 40 mg/kg i.p. of PHCl to normal BDF1 mice.
According to the time-survival curves CBDCA myelotoxicity affects all the tested populations and it is particularly severe on the early steps of hemopoiesis. With the adopted schedule, PHCl does not seem to modify the toxic effects of CBDCA on both peripheral blood cells and hemopoietic progenitors. In spite of these results it is still possible that a protective effect of PHCl on CBDCA myelotoxicity could be observed adopting different schedules of drug administration.

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MEGAKARYOCYTIC PROGENITORS IN PERIPHERAL BLOOD AFTER SALVAGE CHEMOTHERAPY INCLUDING CARBOPLATINUM AND G-CSF ADMINISTRATION IN PATIENTS WITH RESISTANT LYMPHOMA

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Six patients, with resistant or relapsed lymphoma, enrolled in a salvage protocol including chemotherapy with mitoxantrone, carboplatinum, methylprednisolone, cytosine arabinoside (MiCMA) and G-CSF administration, followed by collection of peripheral blood progenitor cells (PBPC) and subsequent transplantation after BuCy2, were evaluated for the presence of megakaryocytic progenitors (BFU-MK and CFU-MK) in a plasma clot assay using IL-3 and GM-CSF as stimulating factors.
In the steady-state condition no colonies were found in PB using unfractionated leukocytes. The colonies became detectable *in vitro*, as immunofluorescent CD41+ cell aggregates, during the second week after chemotherapy and reached the peak levels at day +17+/-2 as CFU-GM and CD34+ cells and in coincidence with leukapheretic procedures. The number of BFU-MK and CFU-MK declined to baseline levels in subsequent days. According to other *in vitro* studies, the addition of SCF did not increase the number of colonies, but influence only their size. In 2 patients, heavily pretreated, megakaryocytic progenitors were undetectable in the recovery phase after MiCMA.
Profound thrombocytopenia (grade 4 WHO) was observed only in this 2 heavily treated patients. In this small series of patients, a faster recovery of the platelet count after myeloablative therapy seems not to be correlated with the dose of megakaryocytic progenitors infused.
Our protocol, although containing high-dose myelotoxic drugs (i.e. carboplatinum), spares uncommitted and committed progenitors and permits a complete and sustained hemopoietic recovery after transplantation.

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ATYPICAL EXPRESSION OF LEUKOCYTE COMMON ANTIGEN (CD45) ISOFORMS ON PERIPHERAL BLOOD PROGENITOR CELLS

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The leukocyte common antigen (LCA or CD45) identifies a family of glycoproteins expressed on the surface of different classes of hematopoietic progenitors (HP): CD45RO is classically detected on more primitive HP (LTC-IC) and on BFU-E; CD45RA characterizes granulo-monocytic and B-lymphoid elements. Data concerning the pattern of expression of CD45 isoforms on CD34+ HP are often controversial. Landsorp et al. found almost equal percentages of CD34+/CD45RO– and CD34+/CD45RA+ cells on normal human bone marrow. Fritsch et al. detected no expression of CD45RO on CD34+ cells from eterogeneous sources; the Authors, however, subdivided the CD34+ population into early CD45RA– and late CD45RA+ HP giving rise, respectively, to compact and dispersed colonies.
To further characterize HP circulating in PB, we carried out a flow cytometric analysis of CD34+ HP mobilized by chemotherapy (CT) and G-CSF in 9 pts affected by lymphoproliferative disease and candidate to peripheral blood progenitor cell transplantation (PBPCT).
Our results show markedly different and non comparable percentages of PB CD34+/CD45RO– and CD34+/CD45RA+ HP ($p=0.0001$, c^2 test). With regard to colony formation, a strong correlation was documented between CFU-GM and both CD45RO+ and CD45RA– early HP (respectively, $r=0.79$, $p=0.0007$; $r=0.81$, $p=0.0004$); on the contrary, no correlation could be assessed for CD45RA+ HP.
In conclusion, in our group of G-CSF+CT mobilized pts, CD45RA could not clearly discriminate between more immature and committed HP.

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COUNTERFLOW CENTRIFUGAL ELUTRIATION OF G-CSF MOBILIZED CELLS: IN VITRO STUDY OF CD34+ CELLS

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CCE has been extensively used to remove T lymphocytes in allogenic BMT. Four peripheral blood mononuclear cell (PBMC) samples collected by apheresis from patients with NHL receiving chemotherapy and G-CSF were elutriated by the CURAME 3000 counterflow elutriator (Heraeus) according to method of Plas et al (*Exp Hematol 1988, 16:355*). Two cell fractions were obtained containing respectively 69±4.2% of monocytes (Fr M) and 76±8.5% of lymphocytes (Fr L). In order to evaluate if functionally different hemopoietic progenitors were obtained in FrM and L, CD34+ cells were isolated from each fraction by immunomagnetic beads as previously described (*Teofili et al. Int J Artificial Organs 1993,16s:89*). CD34+cells were seeded (33,000 /mL) in liquid culture containing Epo 3U/mL+GM-CSF 0.01 ng/mL+IL3 0.1U/mL+SCF 10 ng/mL (for erythroid differentiation) or GM-CSF 10 ng/mL+IL3 100 U/mL+SCF 10 ng/mL (for mye loid differentiation). Total nucleated (Table 1) and clonogenic cells (CFU-GM+BFU-E, Table 2) were evaluated on days 7, 14, 21 and 28.
Results are shown below.

Table 1

	d7*	d14*	d21*	d28*
FrM +Ep	430±273	685±177	1840±802	8809±7823
FrL +Ep	599±510	1296±366	16400±8470	17900±11177
FrM –Ep	726±471	4420±3370	16940±15315	27950±17750
FrL –Ep	716±471	3220±1594	24460±13595	28600±21900

Table 2

	d0*	d7*	d14*	d21*	d28*
FrM +Ep	1,7±0.7	30±20	4.9±3	5.2±1	7.6±7
FrL +Ep	5.6±1	12.9±8	5.9±4	17.7±1	4.9±4
FrM –Ep	1.7±0.7	16.3±9	17.5±9	24.3±23	52.7±52
FrL –Ep	5.6±1	25.3±21	2.8±1	8.2±8	8.5±8

*cells/mL mean number±SEM

Fr M and L show a comparable proliferative activity; clonogenic cell recovery is higher in Fr M. These data indicate that T cell depletion of G-CSF mobilized PBMC can be performed without loss of primitive progenitors.

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EFFECT OF A NEW CHEMOTHERAPEUTIC REGIMEN (MICMA) ON THE MOBILIZATION OF CIRCULATING HEMATOPOIETIC PROGENITORS.

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We employed a chemotherapeutic regimen (MiCMA) associated with G-CSF to mobilize hematopoietic circulating progenitors (HCP) for transplantation in resistant lymphoproliferative disease (NHL and HD). Our protocol consisted of mitoxantrone 10 mg/sm day 1, carboplatinum 100 mg/sm days 1-4, cytarabine 2000 mg/sm day 5, methylprednisolone 500 mg/sm days 1-5, followed by G-CSF (5 ug/kg s.c.) starting from day +8. We evaluated the kinetics of HCP by flow cytometry, determining the frequency and peak of CD34+ cells. Moreover, we calculated the frequency of CFU-GM and BFU-E on days 0, +6, +10, +13 and subsequently every second day.
We found a positive correlation between CD34+ cells and CFU-GM+BFU-E (r=0.76, p= 0.0001), while no correlation was found between BFU-E alone and CD34+ cells and between BFU-E and CD45RO+ cells, which include erythroid progenitors. The peak of CFU-GM and CD34+ cells was observed at a median of 16 days from the start of chemotherapy (range 14-19) and in coincidence with the leukapheretic procedures. The baseline value of CD34+ with respect to mononuclear cells/mL was of 1000±250 cells/mL and in coincidence with the peak of CFU-GM+BFU-E we observed a 9-fold increase of CD34+ cells (9200±2000 cells /mL). The phenotypic analysis of CD34+ subsets revealed that the maximum expansion of both early HCP (CD34+/CD33– and CD34+/HLA-DR-) and late HCP (CD34+/CD33+ and CD34+/HLA-DR+, CD34+/CD38+) occurred at day 16.
These results suggest that: a) our protocol, although containing high-dose myeloablative drugs, spares both uncommitted and committed HCP; b) CD34+ cells correlate with colony forming capacity (CFU-GM+BFU-E) but not with BFU-E; c) G-CSF+MiCMA, the great majority of mobilized CD34+ cells coexpress committment markers such as CD33, HLA-DR and CD38.

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THE IN VITRO ACTIVITY OF SOME URINARY POLYPEPTIDES ON THE PERIPHERAL CFU OF NORMAL AND LEUKEMIC SUBJECTS IN COMPARISON WITH G- AND GM-CSF AND IL3

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Three polypeptides were isolated on HPLC from the acetonic precipitate of the urine of normal subjects and patients with untreated APL or AML. The total polypeptidic extract and the three main fractions obtained were tested on liquid cultures of peripheral CFU of normal and leukemic subjects (AML, APL, CML and CMmL).
The colony growth, the morphologic changes of cells and the main cellular markers were examined at the beginning and after 5 and 10 days of incubation in the medium RPMI 1640, in basal condition of cells and in presence of the single polipeptidic fractions either alone or associated to trans-retinoic acid (R) or thioproline (T), that in previous researches showed a differentiating activity. In the mean time and the same experimental conditions, the activity of G- and GM-CSF and IL-3 were tested.
Results obtained prove the evident colony stimulating activity of at least two fractions and of crude extract, with the ability to modify the behavior *in vitro* of peripheral CFU, sometimes in a similar way to the known growth factors. Other times they show different characteristics, for the ability to stimulate a moderate differentiation of the elements, an increase of fibroblasts formation and of the adhesion molecules.
Are the results expression of a specific growth activity or the consequence of an unspecific ability of several polypeptides to enhance the proliferation and the differentiation of normal and leukemic blasts of the myelomonocytic line?

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COMPARISON OF GM-CSF-INDUCED IMMUNOLOGICAL EFFECTS AFTER CYCLOPHOSPHAMIDE OR CARBOPLATINUM

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The aims of the present study were: 1) to evaluate the biological effects, other than the capacity to mobilize hemopoietic stem cells of GM-CSF; 2) to determine whether these effects are influenced by mono-chemotherapy given prior to administering GM-CSF.
Ten patients affected by multiple myeloma (MM), Hodgkin disease (HD) and low-grade non-Hodgkin lymphoma (LG-NHL) in CR or PR >75% after standard chemotherapy were treated with 7 g/m^2 cyclophosphamide (Cy) and then, at complete resolution of hematological and extra hematological toxicity with 800 mg/m^2 carboplatinum (CBDCA) both followed by GM-CSF 5 μg/kg for at least 16 days. Serum levels of IL-2, but not CD8, soluble receptors increased progressively during GM-CSF infusion IL-1α and IL-1β serum levels were 1.5 higher than basal values after Cy and 2 and >3-fold respectively than basal values post-CBDCA on the 16th day of GM-CSF administration. However, inter-individual values varied more widely after CBDCA. Although tests have not yet been completed, intracellular levels of these two interleukins also seem to rise in response to GM-CSF.
In contrast, no modifications were documented in either serum or intracellular levels of IL-6, IFN-γ and IL-2, except for IL-2 on the 16th post-Cy day. However, the last datum requires confirmation in future tests. Serum neopterin (Np) levels rose during GM-CSF infusion after the administration of both Cy and CBDCA, but the rises were more pronounced and occurred earlier after CBDCA. There were no noteworthy variation in natural killer cell (NK) activity.
Finally, both percent and absolute numbers of lymphocyte populations (T, B, NK) were reduced after Cy, but not after CBDCA. These data suggests that GM-CSF modulates certain T-cell and monocyte-macrophage functions. However, both these functions and other immunological factors are influenced by the chemotherapy given prior to initiating GM-CSF administration.

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PRODUCTION OF GRANULOCYTE-COLONY STIMULATING FACTOR (G-CSF) BY NEOPLASTIC B LYMPHOCYTES FROM B-CLL PATIENTS

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G-CSF is a cytokine with inflammatory and hemopoietic activities released by monocytes-macrophages, fibroblasts and endothelial cells. Recently we have shown that G-CSF is also produced by normal germinal center (GC), but not mantle zone (MZ), B lymphocytes (*Corcione et al., submitted*).
Here we have investigated the ability of purified monoclonal B cells from B-CLL patients to release G-CSF *in vitro*. B cells from 11/22 patients produced G-CSF in 24-48 h cultures (as assessed by ELISA) only upon SAC stimulation, whereas, in 2 additional cases, G-CSF production was observed in the absence of stimuli. CD5+ B cells, which represent the normal counterparts of B-CLL cells, did not release any G-CSF even following incubation with a variety of stimuli.
Studies on the mechanisms of G-CSF production showed that TNF-α was released upon SAC stimulation by all the G-CSF producing B-CLL cell suspensions. Furthermore, incubation of B-CLL cells with a neutralizing anti-TNF-α polyclonal antibody abrogated G-CSF release. Normal CD5+ B cells did not produce TNF-α following *in vitro* stimulation nor did they release G-CSF upon incubation with TNF-α. These results indicate that neoplastic, but not normal, CD5+ B lymphocytes can produce G-CSF *in vitro* and that the synthesis of the cytokine in leukemic cells in absolutely TNF-α-dependent. Whether or not additional cytokines, such as IL1β or TNF-β are also involved in the control of G-CSF gene expression in B-CLL B-cells in now being investigated.

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PRE-HARVEST ABSOLUTE MONONUCLEAR CELL (MNC) COUNT ON BONE MARROW (BM) ASPIRATE CORRELATES WITH MNC YIELD IN AUTOLOGOUS BM HARVESTS

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Autologous bone marrow transplantation (ABMT) has become the treatment of choice for several hematological malignancies. The number of MNC/Kg of body weight (BW) harvested correlates with engraftment, being considered 0.9×10^8/Kg the ideal amount. The aim of our study was to analyze the factors affecting MNC yield in BM harvests in a cohort of patients undergoing ABMT. Data of twenty-five patients (14 NHL, 4 HL, 5 ANLL, 1 ALL, 1 MM) were analyzed.
All the patients underwent a marrow aspirate between 15 and 30 days before harvest, on which the MNC were counted (absolute number/μL). The yield of MNC/kg BW in the harvests was tested in univariate analysis with the following factors: age, sex, diagnosis, previous therapy, interval (days) between end of therapy and BM harvest, state of disease at the time of harvest, MNC count on pre-harvest marrow aspirate. The amount of marrow harvested was considered as a constant, being 21.6 mL±3.7 (SD)/kg BW, (median 21.5 mL/kg BW) with the 95% mean limits of 20.1-23.1 mL. Among all the factors analyzed, only the MNC count on pre harvest aspirate correlated significantly with the MNC yield/kg BW in the harvests (r=0.68; p=0.001).
Although the absolute WBC count of pre-harvest aspirates was higher than that of harvest (due to diluition effect), the MNC percentage was overlapping (p=0.58, Student's t test).
In our patients the mean number of MNC harvested was $0.46\pm0.14\times10^8$/kg BW (median 0.45; range 0.17-0.8); 13 out of 25 patients have already been transplanted: all engrafted with a median time of 15 days to reach PMN $>0.5\times10^9$/L. Data presented show that the MNC count performed on marrow aspirate before harvesting could predict the yield of MNC/kg BW.

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BONE MARROW HARVESTS: EFFECT OF FILTRATION ON HAEMOPOIETIC CELL RECOVERY

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Bone marrow (BM) processing requires a first step of filtration to remove small clots, bone fragments, fat cells and fibrin. This procedure could theoretically cause a significant loss of hemopoietic cells including stem cells. We therefore analyzed the number and the phenotype of cells trapped in the filters and the cellular components recovered after bone marrow filtering.
Five bone marrow harvests from patients with hematologic malignancies were obtained and processed. The following cellular fractions were analyzed: I. whole BM cells; II. fragments trapped in the filters after enzymatic treatement to obtain a cell suspension; III. total BM cells recovered after filtration. Cells from these fractions were counted and characterized by FACS analyzing the expression of CD33, CD34, CD45, CD71.
The percentage of WBC and MNC recovered after filtration was respectively 92.05±5.65 and 88.85±2.80 of the initial population while the percentage of cells trapped in the filter was respectively 3.93±1.25 and 5.65±2.20. FACS analyses performed on this small fraction of cells trapped in the filters showed a high percentage of monocitic-macrophagic cells identified by side scatter and high expression of CD33, suggesting that these typically "sticky" cells non-specifically adhere to the filter. Lymphoid and erithroid sub-populations did not show any relevant difference before and after filtration. Interestingly analysis of CD34+ in fraction II cells showed no specific adherence of stem cells to the filters.
Data presented show that small clots and tissue fragments eliminated by filtration of bone marrow harvests contain a low number of hemopoietic cells with an irrelevant percentage of CD34+ cells. The filtration process therefore does not affect the overall recovery of cells in bone marrow harvests.

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PERFORIN AND GRANZYME B AS PREDICTIVE MARKERS FOR GVHD AFTER BONE MARROW TRANSPLANTATION: PRELIMINARY DATA

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GVHD (*Graft Versus Host Disease*) represent an important problem for the success of an allogeneic bone marrow transplantation (BMT). For donor recipient matching the use of the mixed lymphocyte cultures (MLC) is generally unhelpful to predict the disease. Actually the only method validated to prognosticate the GVHD is the analysis of cytotoxic T-lymphocyte precursors but this tecnique requires the support of radioactive elements not suitable in all the laboratories. It is known that T-cells implicated in the early skin lesion after BMT) express serine proteases such as granzyme B and perforin. In our study we used a cytochemical method to detect the cytolytic cell-specific lymphoid serine protease (granzyme B and perforin). Using N benzyloxycarbonyl-L-lysine thiobenzyl ester (BLT) as the serine esterasi (*Wagner, Sunder-Plassmann et al., J Immunol Methods 160,173-80, 1993*) we have evaluated the presence of cytoplasmatic granules in lymphocytes from MCL (*Dacie L., Pract Hematol, 1984*). The serin proteasi activity (SP) has been demonstrated by incubation at 37°C for 10 min using 2×10^{-4} M BLT as substrate in 0.2 M Tris-HCL buffer pH 8.1 incorporating 0.2 mg/mL Fast Blue BB (Sigma) as a chromogenic capture agent. Then slides are counterstained with Harris' hematoxylin.
In two out 6 cases analyzed in our laboratory we have observed a significant presence of SP-granule-bearing cells. One of the two patients was submitted to BMT and then showed a chronic hepatic GVHD.
In this case we have found SP positivity in cells obtained from the condition donor+recipient of the MLC. Our data are preliminary, but they suggest, if they will be confirmed, that this method could be used as early marker to predict GVHD.

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A RAPIDE PROGRESSIVE PULMONARY FAILURE IN A YOUNG PROMYELOCYTIC LEUKEMIA PATIENT AFTER ALLOGENEIC BONE MARROW TRASPLANTATION

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The old male 16-yrs patient with acute promyelocytic leukemia received an allogeneic bone marrow from his HLA-ABO identical, MLC compatible 17-year-old sister. For GVHD prevention the patient received a combination treatment of 2 mg/die methylprednisolone and 25 mg/week methotrexate. It was not possibile to use cyclosporine because of its neurotoxicity early revealed by the patient. On day 189 the patient presented with a rapidly progressive dyspnea, non-productive cough, bilateral inspiration crackles and wheezes, subcutaneous neck emphysema in consequence of pneumomediastin. Pulmonary function tested showed a severe restrictive and obstructive defect with no change after inhaled broncodilators. Chest Rx was negative. Chest computed tomograph showed little multiple bilateral areas of air traffing and areas of alveolar capacities with vascular call thickneing and peribronchial infiltrative. No microorganism was proved responsible of these lung failure. Because of the worseningof clinical conditions from 238 day after transplant the methotrexate was replaced by cyclosporine (5 mg/Kg/day); also with this treatment the clinical conditions of the patient didn't improve but it became stable. In consequence of a hypercapnic crisis and a following respirations block for hypercapnic coma, it was necessary a tracheotomy with a positive ventilation. Soon after from 500 days after transplant the clinical conditions, vital parameters and pulmonary tests became stationary. However in consequence of a severe pneumotorax on day 726 after BMT, the patient died.
We believe that the only diagnostic test for early recognition of a common sort of chronic GVHD was spyrometric and hemogasanalyze. These lung function studies are so important that, in accordance with some authors, we suggested not transplanting a patient with FVC, FEVI <75%.

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"FINGERPRINTING" OF HLA-CLASS I AND II GENES FOR IMPROVED SELECTION OF RELATED AND UNRELATED BONE MARROW DONOR

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The grade of matching of the HLA genes between selected donor and recipient is an important problem in the selection of unrelated donors for allogeneic bone marrow transplantation (UBMT). Serological screening of HLA-class I genes, mixed lymphocyte culture (MLC), and molecular genotyping of HLA-class II genes are the more sensitive methods currently used. Furthermore, serological screening of class I antigens (A,B,C) fail to detect minor diversities, which, in contrast, have been demonstrated by direct sequencing of DNA polymorphic regions. Molecular genotyping of HLA-class I genes by DNA analysis is money-expensive and it takes a lot of work. To improve compatibility between donor and recipient, we have set up a new rapid and not-radioisotopic application of the *fingerprinting-PCR* technique, to the analysis of the polymorphic second exon of HLA-class I A, B, C genes. The technique is based on the formation of specific patterns (fingerprints-PCR) of homoduplexes and heteroduplexes between heterologous amplified DNA sequences. After electrophoretic run on not-denaturing polyacrylamide gel, different HLA-class I types give allele-specific banding patterns. HLA class I matching is defined after ethidium bromide soaked or after silver staining by visual comparison of patients' fingerprints with donors' ones. Identity can be confirmed by mixing donor and recipient DNAs, in an amplified crossmatch. To assess the technique we analyzed: normal samples, 22 related allogeneic bone marrow transplanted pairs, and 10 unrelated HLA-A and HLA-B serological matched patient-donor pairs were analysed for HLA-class I polymorphic regions. In all the related pairs and in one out of ten unrelated pairs not-mismatched different donor-transplanted patterns were identified. This new application of PCR fingerprinting may confirm the HLA-class I serological selection of unrelated marrow donors.

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EFFECT OF BONE MARROW TRANSPLANTATION ON THE DAYTIME MELATONIN CIRCULATING LEVELS

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Several experimental observations seem to suggest a relation between the pineal gland and neoplastic growth. It has been demonstrated that pinealectomy enhances tumor growth and metastases, while the administration of melatonin (MLT), may inhibit the growth of same forms of cancer. Therefore, the clinical significance of MLT blood level measurements in the prognosis of human neoplastic diseases is still unclear and has still to be established. Preliminary observations, however, seem to show that MLT secretion changes in relation with the tumor mass, since a marked decrease in its levels has been reported after surgery in women with breast cancer and in a group of oncological patients after chemotherapy.
We evaluated MLT serum levels (pg/mL) in 19 patients with hemolymphopoietic neoplasm addressed at the autologous or allogenic bone marrow transplantation, at baseline, after conditioning regimen and 3 months after. 19 age matched healthy subjects (39.1±9.1 versus 37.9±14.6; $p = 0.8$) were studied as controls. These preliminary data concerning only first and second time. Oncological patients showed higher MLT levels than controls at baseline (28.3±10.2 versus 12.5±3.4; $p < 0.001$); after conditioning regime MLT fall in a significant way (28.3±10.2 versus 16.9±8.4; $p < 0.001$). The reduction of MLT levels may be a consequence of a direct damage at the pineal level induced by chemotherapy or radiotherapy or due to reduction of the tumor mass. However, MLT levels not differ from patients subjected at total body irradiation and/or chemotherapy agents which cross the blood-brain barrier (7 patients) and others (12 patients) (14.7±11.1 versus 18.2±6.6; $p = 0.39$).
Since high levels of MLT could produce a down regulation of its receptors, the fall of MLT after conditioning regime may stimulate the activity of the hormone or represents a diminished stimulus from tumor on the pineal gland. The MLT decreases after conditioning regime should be regarded as an undesiderable effect or simply as a marker for the effectiveness of the treatment.

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G-CSF AND IL-3 COMBINATION ACCELERATES HEMATOPOIETIC RECOVERY AFTER ABMT FOR LYMPHOMA PATIENTS

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We have treated 19 consecutive lymphoma patients (pts) submitted to autologous bone marrow transplantation (ABMT) with the growth factors (GFs) combination scheduled as follows: G-CSF (5 g/Kg/day s.c.) from day +1 and IL-3 (10 g/Kg/day s.c.) from day +6. Both GFs were discontinued after granulocyte recovery (>500 ANC/L). The results obtained were compared with a historical control group of 22 pts who received G-CSF alone. The 2 groups were comparable as for age, number of previous chemotherapy lines, number of mononuclear cells and CFU-GM reinfused. Three pts in the G-CSF/IL-3 group were reinfused with bone marrow (BM) purified CD34+ cells obtained by CEPRATE LC Stem Cell Concentrator (Cell Pro).
The hematological recovery was significantly faster for pts (= 16) receiving the GFs combination ($P < 0.05$ for all the comparison data):

	G-CSF/IL-3	G-CSF
Day ANC >200/L	9 (8-18)	12 (8-26)
Day ANC >500/L	11 (9-29)	13 (10-29)
Days <100 ANC/L	4.5 (3-9)	7 (3-16)
Days <200 ANC/L	7 (4-14)	10 (5-17)
Day >20.000 PLT/L	15 (9-27)	19 (12-300)
Days <20.000 PLT/L	9.5 (2-23)	15 (7-200)

We also observed a lower PLT trasfusion requirement and shorter hospitalization.
Hematopoietic reconstitution of pts who received purified CD34+ cells did not significantly differ from that of the individuals reinfused with unmanipulated BM cells. Parallel, *in vitro* studies have demonstrated that the multilineage response to G-CSF/IL-3 seems to be dependent upon stimulation of multipotent and lineage restricted progenitor cells.
In summary, G-CSF/IL-3 combination is safe and well tolerated in lymphoma pts after ABMT and enhances granulocyte and platelet recovery.

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SALVAGE CHEMOTHERAPY AND G-CSF ADMINISTRATION FOLLOWED BY AUTOLOGOUS PERIPHERAL BLOOD PROGENITOR CELLS TRANSPLANTATION IN PATIENTS WITH RESISTANT LYMPHOMA

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We enrolled 29 patients affected by refractory or resistant lymphoma (5 HD,24 NHL) in a two-steps protocol including salvage chemotherapy with mitoxantrone, carboplatinum, methylprednisolone and cytosine-arabinoside (MiCMA) plus G-CSF (5 ug/Kg/d), peripheral blood progenitor cell (PBPC) collection and subsequent transplantation after BuCy2 regimen. After MiCMA 4 patients (14%) achieved complete response (CR), 17 patients (60%) partial response (PR) and 6 showed progression of disease (PD). Eighteen patients completed the protocol. Three patients in CR, although eligible, refused PBPCT and they are in continuous CR at 26, 24 and 26 months, respectively. One patient, in PR after MiCMA, received mantle field irradiation, she achieved CR and was not considered eligible for transplant. All transplanted patients were evaluable. Eight patients (44%) are currently alive in CR with a median follow up of 21.5 months (range 5-29); one patient remains in PR 3 months after PBPCT. Three patients are currently alive but in relapse after 15 months from PBPCT. One patient relapsed after 4 months from PBPCT, achieved further CR after α-interferon treatment. Hematological reconstitution was very rapid with a median time to achieve PMN $>0.5\times10^9$/L and plts $>50x10^9$/L of 12 (range 9-18) and 10 (range 0-36). No hemopoietic growth factors were administered after PBPCT. Eleven of these transplanted patients showed a transient fall in platelet count, nearly after 60 days from PBPCT, followed by a prompt rise without supportive care.
Our protocol seems to be very effective as a salvage treatment meanwhile permitting PBPC collection after G-CSF administration. Hemopoietic reconstitution after transplantation is complete, rapid and self-substained.

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IMMUNOCYTOCHEMICAL AND IMMUNOELECTRON-MICROSCOPICAL IDENTIFICATION OF P-170 IN RAT PERITONEAL MAST CELLS

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P-glycoprotein or p-170 is a 170 kDa transmembrane glycoprotein which is overproduced in tumor cells exhibiting the multidrug resistance (MDR) phenomenon. P-170 has been identified also in normal cells actively involved in absoptive/secretive processes, such as the epithelial cells of the large intestine and renal tubules, the exocrine pancreatic cells, the hepatocytes, the endometrial cells and the endothelial cells at blood-tissue barrier sites. Previous work in our laboratory has shown that adriamycin (ADR) exhibits a tremendous affinity for rat peritoneal mast cells and that this drug is highly concentrated in the mast cell granules. The mechanism of ADR accumulation is strictly related to the process of mast cell degranulation and seems to involve an active trasport system which closely resembles the energy dependent outward transport system of MDR cells.
The aim of the present study was to demonstrate the presence of P-170 in rat peritoneal mast cells and to try to localize this protein at the ultrastructural level.
Mixed peritoneal cells were obtained by lavage of the peritoneal cavities of Sprague-Dawley rats with saline solution at 4°C.
Immunocytochemistry was performed with the APAAP method on cytospin preparations using MRK-16 and JSB monoclonal antibodies (mAbs). The immunofluorescence procedure and immunoelectron-microscopy were carried out on cell suspensions using the same mAbs. For the ultrastructural localization of P-170 the protein A-colloidal gold method was used.
Immunocytochemical and immunofluorescence findings showed that P-170 was primarily concentrated in mast cell granules; little amount of P-170 immunoreactivity was localized on the plasma membrane. In some experiments the fluorescence signal was found to decorate the granule-coating membranes. Electron-microscopical observations revealed a marked accumulation of colloidal gold particles on the surface of the secretory granules wherease decoration of the plasma membrane was less intense.
These results demonstrate that P-170 is expressed in normal rat peritoneal mast cells. The localization of this protein on the surface of mast cell granules suggests its possible implication in the mechanisms of the transmembrane transport for various molecules. These data are to be considered as the initial step for further morphological and pharmacological studies concerning the process of ADR internalization in mast cells.

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EVALUTATION OF MULTI-DRUG RESISTANCE IN PEDIATRIC LEUKEMIAS

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One of the most studied mechanisms of resistance to cytotoxic drugs in cancer cells is the multidrug resistance [MDR 1]. MDR is frequently associated with the expression of a membrane glycoprotein termed P-glycoprotein or GP170.
Many malignancies may initially be chemoresistant or acquire drug resistance after exposure to cytotoxic therapies This represents one of the major problems in cancer treatment. With this study we tried to achive a qualitative and quantitative analysis of MDR.
To evaluate the uptake and the retention or release of MDR correlated drugs we used the rhodamina 123 [Rhd-123]. Rhd-123 is a fluorescent dye transported by the P-glycoprotein.
We studied pediatric patients with leukemia either at diagnosis, prior to any treatment, or in relapse.
MDR was detected more frequently in relapse than at diagnosis. Patients with MDR showed great difficulties to gain the complete remission. In order to distinguish whether this is due to a selection of resistent cells or to drug induced resistance, two cell lines, obtained in our laboratory from two different patients affected by a solid tumor with bone marrow infiltration, were analyzed.
Both cell lines were drug sensitive, without presence of MDR. These cells, initially drug sensitive, were exposed to low dose chemoterapy to evaluate if drug resistance may be inducted.
Preliminate data seem to confirm that cell tumors, initially drug sensitive, after exposition to chemoterapeutic drugs may develope MDR. The acquisition of resistance seem to be directly correlated to duration and concentration of drug exposure.

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CLINICAL RELAVANCE OF GLUTATHIONE-S-TRANSFERASE AND MULTIDRUG RESISTANCE IN B-CLL

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Lymphocytes from patients affected by B-CLL have been shown to be frequently MDR positive. However, this phenotype do not seems to be responsible for the resistance to alkylating agents that are usually emphoyed in the management of CLL. On these basis we evaluated in lymphocytes from 42 patients the expression of P-170 and the activity of glutathione-s-transferase (GST) that is a possible candidate for the resistance to chlorambucil.
The present results show that GST is not related to any clinical parameter but it was increased in treated patients. Conversely 85% of patients were positive for P-170 and this was related to the percentage of CD5/CD19 positive lymphocytes. CD5/CD19 negative patients were negative for P-170 too.
MDR does not related to any clinical parameter evaluated nor to GST activity in lymphocytes.

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INTERLEUKIN-2 AND α-INTERFERON MODULATION OF CHLORAMBUCIL- AND PURINE ANALOGS-INDUCED CYTOTOXICITY IN CHRONIC LYMPHOCYTIC LEUKEMIA

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The *in vitro* cytotoxic effect of chlorambucil (CLB), fludarabine (FAMP), and 2-chlorodeoxyadenosine (CDA) on peripheral lymphocytes from 49 previously untreated CLL patients has been studied by a MTT colorimetric assay, evaluating recombinant (r)IL-2 and α-IFN effect on drug-induced cell death.
The results of these experiments showed that rIL-2 significantly increased CLB ID50 (drug concentration able to kill 50% of cells) value (median value 17.97 μM versus 172.02 μM, p=0.0023), while purine analog cytotoxicity was rescued by the cytokine with a borderline significance.
The potential *in vivo* relevance of rIL-2, which behaves as a survival signal on CLB-induced cell mortality, is also suggested by the correlation found between the lowest IL-2 serum levels, *in vitro* sensitivity to CLB, and achievement of clinical complete response after CLB treatment in 8 CLL patients. In the MTT reduction assay, α-IFN allowed CLL cells to become more resistant to CLB, CDA and FAMP in 14, 8 and 7 out of 25 samples, respectively; conversely, α-IFN synergized with both CLB and FAMP in 6 samples and with CDA in 4 cases.
These results are in line with our data of immunoenzymatic assays showing that α-IFN either up- or down-regulated tumor necrosis factor (TNF) and IL-1 levels in supernatants of 11 CLL samples cultured for 4 days. The results of this study indicate that α-IFN can play a dual role in regulating drug-induced cell death, while the mechanism of action of IL-2 seems to be only in favour of cell survival in CLL.

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MDR-RELATED P170 GLYCOPROTEIN MODULATES THE ANTILEUKEMIC ACTIVITY OF HOMOHARRINGTONINE

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The identification of homoharringtonine (HHT) as a drug active against leukemic cells led its use in the therapy of acute myeloid (AML) and lymphoid (ALL) leukemias, either alone or in combination with other agents. Since HHT showed a low efficacy in the refractory and relapsed leukemias and in the blastic phase of chronic myeloid leukemia (CML) which are frequently characterized by a high P170-expression we supposed a relationship between the antileukemic effect of HHT and the expression of P170 glycoprotein. For that purpose, sensitive (LOVO 109 and CCRF CEM) and MDR (LOVO DX and CEM VLB) cell lines were exposed to HHT with or without the following MDR modifiers: cyclosporin A (CyA), SDZ PSC 833 and Dx-verapamil.
We observed that MDR cells were about 15-folds more resistant to HHT than non-MDR cells and that resistance to HHT was significantly decreased by all the MDR modifiers that were tested. Our results show that HHT belongs to the category of MDR-related drugs, like anthracyclines, vinca alkaloids, and epipodophylline derivatives.

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TREATMENT OF ACUTE MYELOID LEUKEMIA WITH CYCLOSPORINE A PLUS CHEMOTHERAPY

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Drug resistance is the major cause of treatment failure in acute myeloid leukemia (AML) and is frequently associated with the overexpression of the multidrug transporter, P-glycoprotein (P-170). This type of multidrug resistance (MDR) can be effectively downmodulated, *in vitro*, by cyclosporine A (CsA) at concentrations ranging between 1 and 3 μM. The objective of this phase-1 study was to characterize the combined effects of CsA and chemotherapy (CHT) in 21 patients (pts) with AML. The median age was 57 years (range 18-64 years). Cytotoxic treatment was based on arabinosyl cytosine (AC), standard or high dose, plus idarubicin or mitoxantrone and etoposide. CsA was given through a continuous i.v. infusion (CI) for 72 to 106 h. In 23 courses the CsA dose was 10 mg/kg/day and a median CsA serum steady state concentration of 0.9 μM (range 0.6 to 1.4 μM) was obtained. In 4 courses the CsA dose was 12.5 and a median CsA serum steady state concentration of 1.1 μM was obtained. Finally, in 2 courses the CsA dose was 15 mg/kg/day and a CsA serum steady state concentration of 1.6 and 1.7 μM were respectively achieved. A complete remission (CR) was obtained in 6/9 cases at onset, in 2/9 cases in relapse and in 2/3 cases with resistant disease. 7 failures and 4 early deaths were observed. No pt, at onset, was refractory to induction treatment. All responders had MDR leukemic cells. P-170 expression was analyzed by flow cytometry and immunocytochemistry using the alkaline phosphatase anti alkaline phosphatase (APAAP) technique, with the MRK16 monoclonal antibody. This analysis was performed on all of the pts before starting therapy and at the moment of leukemia regrowth.
Toxicity and side effects included weight gain (> 5%) in 10/29 courses, hypertension (diastolic pressure > 100 mmHg) in 9/29 courses, vomiting in 8/29 courses (grade III 2/8), grade III stomatitis in 8/29 courses, and grade III enterocolitis in 4/29. Reversible hyperbilirubinemia, without other liver function abnormalities, was observed in 13/29 courses during CsA CI. The increase of bilirubin was correlated with the plasma CsA level (r=0.615 p=0.002). The rate of CR was lower, but not significantly, in pts who experienced hyperbilirubinemia. For those who responded the median time to reach 0.5×10^9/L neutrophils was 26 days (range 22-32 days) and 50×10^9/L platelets was 29 days (range 24-40 days). Serum creatinine concentration was normal before treatment (median 8 mg/L, range 7-13 mg/L) and did not change during or after treatment. Early deaths included CNS hemorrhage (3 cases) and infection (1 case).
In conclusion, our preliminary data suggests that 1) CsA could be reasonably combined with intensive CHT without reducing the optimum dose of cytotoxic drugs, 2) hemorrhages and mucosal toxicity may play a major dose-limiting role, 3) to reach a serum concentration close to 2 μM, which has been successful *in vitro*, a higher dose of the modifier agent should be used.

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INCIDENCE OF P170 EXPRESSION IN ACUTE AND CHRONIC LEUKEMIAS

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From 1989 to 1994 we investigated the MDR phenomenon in 156 cases of acute non lymphocytic leukemia (ANLL), 41 cases of acute lymphocytic leukemia (ALL) and in 43 cases of chronic myeloid leukemia (CML).
We assayed the expression of P170 glycoprotein using the MRK16 monoclonal antibody with the immunocytochemical technique APAAP. We defined positive only the cells sharing the same positivity of MDR positive cell lines.
In ANLL we found positive cells in 39/96 (40%) cases at onset and in 30/60 (50%) cases at relapse (median 6 and 17 respectively). 71/96 (74%) cases were evaluable for the outcome of therapy: among these a complete remission with a first line chemotherapy schedule was achieved in 29/51 (56%) patients with positive cells and in 16/20 (80%) without positive cells (NS). This therapy failed in 20/51 (39%) cases with positive cells and in 4/20 (20%) cases without positive cells (NS). Relapse occured in 22/29 (75%) patients with positive cells and in 7/16 (35%) patients without positive cells (p=0.02). No significant differences were found in survival between patients with and without positive cells, but complete remission was remarkably longer in patientw without positive cells (p=0.04).
As far as ALL are concerned, positive cells were found in 18/27 (67%) cases at the onset, and in 11/14 (78%) cases at the relapse. 22/27 (81%) cases received the same first line chemotherapy and were evaluable for the outcome: complete remission was achieved in 10/14 (71%) patients with positive cells and in 7/8 (87%) without positive cells. Primary resistance occured in 7/10 (70%) and in 2/7 (28%) patients with a without positive cells respectively.
In CML, in contrast with previous data, we found a variable percentage of positive cells in all phases of the disease, with an higher number of positive cells in blastic phase cases (p=0.006). Positive cells were found in 3/26 (11%) blastic phase cases and in 6/17 (35%) chronic phase cases.
These data underlines the role of P170 overexpression in hematological malignancies and warrant clinical trials with MDR modifiers.

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COMPARATIVE TUMORICIDAL ACTIVITY OF IDARUBICIN AND IDARUBICINOL IN COMBINATION WITH CYCLOSPORIN A IN MDR LEUKEMIA CELLS

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Idarubicin (IDA) is a 4-demethoxydaunorubicin analog with a superior *in vitro* cytotoxicity against tumor cell lines compared with daunorubicin. It shows a low degree of cross-resistance when tested against doxorubicin-resistant and multidrug resistant (MDR) cell lines. IDA is converted in the liver in idarubicinol (2H-IDA) and, in this form, seem to exert your antitumoral activity *in vivo*.
Recent works showed that 2H-IDA has tumoricidal activity similar to that of the parent drug when tested *in vitro* in sensitive neoplastic cells. We compared *in vitro* the activity of IDA and 2H-IDA in MDR cell lines K562/R, CCRF-CEM VBL, RFLC, and in their parent sensitive cell lines. IDA and 2H-IDA show the same cytotoxic activity in sensitive cells. This activity is about 1Log higher than that showed by daunorubicin (DNR). After 1h exposition to each drug we observed that also the cellular uptake of IDA and 2H-IDA is similar. In resistant cells 2H-IDA is about 0,5Log less active than IDA and your cytotoxic activity is intermediate between that showed by IDA and DNR. We observed that the intracellular uptake of IDA is lower than that of 2H-IDA and this can be correlated to a greater ability of P-glycoprotein to expel 2H-IDA than IDA. In fact, if MDR cells are exposed to IDA and 2H-IDA in combination to 2 uM cyclosporin A (CyA) the cytotoxic effect of the two anthracyclines results the same, similar to that observed in sensitive cells, and 1Log higher than that obtained using DNR in combination to 2 uMCyA.
These data confirm the utility of the combination between IDA and a MDR reversing agent in hematological malignancies displaying the MDR phenotype.

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MODULATION OF MULTIDRUG RESISTANCE (MDR) BY L(GR66235A) AND R(GR66234A) ENANTIOMERS OF TELUDIPINE, A NEW DIHYDROPYRIDINE CALCIUM CHANNEL BLOCKER

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Many dihydropyridine analogues with calcium channel blocker activity are able to reverse multidrug resistance (MDR). Some of these compounds are more effective than verapamil in enhancing the antitumoral activity of anthracyclines and vinca alcaloids *in vitro*. Teludipine is a new dihydropyridine calcium channel blocker synthesized by Glaxo research center and characterized by high lipophilia and low cardiotoxicity. R-enantiomer (GR66234A) of teludipine has a calcium channel blocking activity about 100 times *in vitro* and 10 times *in vivo* lower than L-enantiomer (GR66235A). We studied the daunorubicin (DNR) resistance reversing activity of GR66234A and GR66235A in two MDR cell lines: ARNII (murine erythroleukemia), in which the MDR is correlated to high levels of P-glycoprotein (Pgp), and Adr MCF-7 (human breast cancer) where the MDR is correlated to high levels of Pgp and high glutathione-S-transferase (GST) activity. GR66234A and GR66235A show identical activity in reversing DNR resistance, and are more active than verapamil. In ARNII cells, 0.15 uM GR66234A shows a DNR reversing activity similar to 1 uM verapamil. In Adr MCF-7 cells the difference is less marked, and 0.4 uM GR66234A have about the same activity than 1 uM verapamil. Enantiomers of teludipine at concentration of 5 uM determine a DNR accumulation on MDR cells which is similar to that obtained on the sensitive cells and greater than that observed with verapamil. The difference in DNR accumulation between verapamil and teludipine is lower when they are used at concentration of 1 uM. No difference in intracellular DNR distribution has been observed between teludipine and verapamil. The discrepancy between cytotoxicity data and DNR accumulation for concentration of teludipine lower than 1 uM led to hypothesize others mechanisms different from the inhibition of drug accumulation for the MDR reversing activity.

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THE PERSPECTIVES OF IN VITRO PURGING WITH ETOPOSIDE AND IDARUBICIN: PRELIMINARY RESULTS

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The clinical impact of the bone marrow purging is already under investigation and its efficacy has been reported only in retrospective overview with cyclophosphamide derivates in acute non lymphoblastic leukemias. One of the most important critical point is that the dose-response curve of maphosphamide (ASTA-Z) is unpredictable; moreover the log-kill of tumor cells could be improved by introducing or adding new drugs, with improved therapeutic index. Our experimental model used two different targets: normal bone marrow cells and leukemic lines, in order to compare the efficacy of idarubicin (IDR) and etoposide (VP16). We used three leukemic linees: U-937, K-562 and CEM. First, we evaluated the clonogenic efficiency of each line in a semisolid assay; second the inhibition of their growth in methylcellulose has been tested by using different concentrations of the two drugs. Clonogenic efficiency of the three lines was 54% for K562, 15% for CEM and 13% for U-937. Growth of the leukemic lines has not been abolished by incubation with VP16 at the concentration of 60 μM while the concentration of 120 μM proved to inhibit only U-937 line; in fact for both K-562 and CEM line we observed a residual growth even at this concentration (respectively 4.2±3.3% and 1.8±2.6%). IDR, at the concentration of 0.05 μg/mL totally inhibited the growth of U-937 and CEM, while K-562 proved to be much more resistant; indeed at the concentration of 5 μg/mL we observed a residual growth of 22.9±6.3%; only the concentration of 10 μg/mL completely abolished K562 growth. Basing on these results we tested the K562 line for three different concentrations of ASTA-Z (25, 50, 100 μg/mL), obtaining a complete growth inhibition at the intermediate concentration of 50 μg/mL. Contemporarily the growth of normal hematopoietic progenitors has been tested in semisolid assay, comparing the toxicity of VP16, IDR and ASTA-Z. In summary the incubation with VP16 induced a progressive inhibition of CFU-GM, but with a residual growth of 14% at the maximum concentration of 120 mM. IDR showed a stronger toxicity because at the concentration of only 0.1 μg/mL we could observe a residual CFU-GM growth of 17.8±3%. ASTA-Z showed the most predictable dose-response effect sparing the hematopoietic progenitors even if used at the concentration of 100 μg/mL. In conclusion VP16 showed the optimal efficacy against the monoblastic line U-937; the lymphoid line CEM and the erythroleukemic K-562 cells proved to be resistant even at the maximum tolerated dose for normal hematopoietic progenitors used. ASTA-Z showed best activity on the K562 line; on the contrary this line was completely resistant to IDR; indeed this resistance could be overcome only by using lethal concentrations for normal bone marrow progenitors.
Finally, this drug showed a complete inhibition both of U-937 and CEM line proliferation at the 50% of the drug concentration, sparing 18% of normal CFU-GM.

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PHASE II CLINICAL STUDY OF DEXVERAPAMIL PLUS VAD FOR THE TREATMENT OF ANTHRACYCLINE-REFRACTORY MULTIPLE MYELOMA (MM)

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We report the results of an open label, phase II clinical study investigating the tolerability and efficacy of VAD and associated Dexverapamil (R-VPM) (Knoll AG, Ludwigshafen/Germany) as chemosensitizer for patients with MM refractory to previous VAD or novantrone-containing regimens. VAD was given by continuous i.v. infusion on d 1-4 of each cycle. R-VPM, the R-isomer of verapamil, was administered orally at the dose of 180-240 mg/sqm every six hours, starting on d O and continuing until d 6 of each cycle. To now, 9 patients (median age 62 yrs; IgG type 7, Bence Jones type 2; stage I 2, stage II 2, stage III 5; MDR-1 positive 4, MDR-1 negative 5) entered the study. Side effects of R-VPM were generally mild and consisted of bradycardia and/or hypotension. No patient required pressor support or other cares. In 3 patients adverse reactions were related to the increase in the dose of R-VPM which, in 2 of them, was subsequently de-escalated to the lower dose. However, in 1 patient R-VPM had to be discontinued because of persistent bradycardia. One other patient was withdrawn from the study after the first cycle because of cardiac ejection fraction <45%. The remaining 7 patients received at least 2 cycles of therapy, 6 of them at the higher R-VPM dose of 240 mg/sqm on the second cycle. Five patients who had been refractory to at least three courses of VAD administered immediately before the start of the study were analyzed for response to VAD+R-VPM. Of them, 1 patient (MDR-1 positive) had more than 75% decrease in M-protein production rate, while the remaining 4 (MDR-1 positive 3; MDR-1 negative 1) had no change during therapy. Pharmacokinetics of R-VPM and its active metabolite, Norverapamil (N-VPM), was performed in all patients during the first course of therapy. The highest mean serum concentration of both these compounds was reached after 40 minutes from R-VPM administration and was 1082 ng/mL (2.2 μM). However, plasmatic levels of R-VPM and N-VPM did not seem to be steady-state during therapy. The only patient showing an objective response had the highest mean serum concentration of both these compounds (1780 ng/mL or 3.5 μM), which remained constant until 180 minutes from R-VPM administration. In conclusion, preliminary results of this study show that oral R-VPM at the dose of 180/240 mg/sqm can be administered relatively safely even to elderly patients with MM. Further analyses are warranted to establish the ideal schedule of administration of R-VPM and its role as chemosensitizer in the management of patients with anthracycline-refractory MM.

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ATRA SENSITIVE SKIN RELAPSE OF ACUTE PROMYELOCYTIC LEUKEMIA

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Acute promyelocytic leukemia (APL) is characterized by the specific traslocation (15;17). All-trans-retinoic acid (ATRA) as single drug is able to induce transient complete remission (CR). Extramedullary localizations of APL are uncommon. We report a patient with cutaneous relapse of APL, which has been controlled and mantained by ATRA during the past two years.
A 31-year-old woman received diagnosis of APL in August 1990, with typical hypergranular blasts and t(15;17). Treatment was started according to the GIMEMA LAP 0389 protocol (induction arm IDA+ARA-C) and CR was achieved in September 1990. The protocol was completed in December 1990. In March 1992, while in persistent hematological CR, a few little cutaneous nodules (< 2 cm) appeared on her trunk; a leukemic blast infiltration was demonstrated histologically. DNA analysis (Southern blot) of the biopsy revealed the hybrid PML/RAR gene, which was confirmed by reverse PCR (RT-PCR) of its transcript. A two-month treatment with ATRA (70 mg/day) caused disappearance of the nodules. In July 1993 they reappeared, some of them in the previous sites: a 3-month ATRA treatment was again effective. Two months after ATRA withdrawal, skin relapse occurred once again and treatment was restarted, followed by a new remission that is now mantained with daily administration of the drug. The search for PML/RAR transcript on bone marrow cells by RT-PCR has always remained negative.
Cutaneous involvement is uncommon in APL. Local trauma (e.g. insertion of central venous catheter), reported by others as a cause of skin localization, was absent in our case. ATRA caused disappearance of the cutaneous nodules, but a continuous administration was required to mantain the results. Patient's bone marrow has been recently harvested and an autograft is programmed.

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ALL-TRANS RETINOIC ACID (ATRA) IN THE TREATMENT OF MYELODYSPLASTIC SYNDROMES (MDS)

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Myelodysplastic syndromes (MDS) are a group of hematopoietic disorders characterized by bone marrow uni- or multilineage maturation defects. Controversial results have been obtained using growth factors or differentiating agents such as 13-cis retinoic acid in the treatment of MDS.
In this study we evaluated the effects of all-trans retinoic acid (ATRA) in 10 MDS patients (5 male, 5 female). Six patients had refractory anemia (RA), 1 had refractory anemia with excess of blasts (RAEB), and 3 had refractory anemia with excess of blasts in transformation (RAEB-t). All the patients received the same dose of ATRA (45 mg/sqm/day) orally for 6 weeks. A rise in hemoglobin concentration > 1 g/dL was observed in 3/10 patients, 5/10 patients showed an increase in granulocyte count > $0.5x10^9$/L without concomitant increase in the percentage of blast cells in the bone marrow. A rise in platelet count > $50x10^9$/L was observed in 1/10 patients. All the effects were transient, maximal responses were obtained by the second or third week of treatment, after which time peripheral blood counts started to drop again, reaching pre-therapy values by the end of treatment.
This phenomenon could be attributed either to the exhaustion of an ATRA-responding cell pool, or to cellular resistance to ATRA or to a reduction of plasmatic ATRA levels after prolonged treatment. According to our results, however, ATRA might hold promising therapeutic efficacy in MDS, especially if its effects could be improved by combinations with other differentiating agents or growth factors.

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RESISTANCE TO ATRA IN APL AT DIAGNOSIS: DESCRIPTION OF A CASE

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All transretinoic acid (ATRA) has become a useful agent for the treatment of acute promielocic leukemia (APL), being capable of inducing a complete remission rate of 80-90%, also in patients refractory to or unsuitable for standard chemotherapy. However, we observed a case of primary resistance to ATRA in an elderly woman with APL.
Our patient, a eighty-three old woman, was admitted to ward with leukocytosis (WBC 93000/L), trombocytopenia (Plts 24000/L), anemia (Hb 8.9 gr/dL) and abnormalities of coagulation: PT: 60% fibrinogen 190 mg/dL and D-Dimer elevated (>4 g/mL). Also presented with a severe ischemia of right foot.
The diagnosis of APL was performed on the basis of both morphology and citochemical stain of bone marrow.
Therapy was started with ATRA 100 mg/day. Because of the high leukocyte number we also gave a single dose of 1 g ARAC. Leukocytes dropped, however, leukemic cells were not cleared froom blood. D-Dimer persisted slightly elevated. After three weeks circulating leukemic cells abruptly increased up to 27,000/L. A second ARAC dose 1 g was given but the patient died shortly after because of intracranial bleeding.
APL resistance to ATRA has been usually observed in plurirelapsed patients. In this case no significative response at all was observed at diagnosis.
In our case, however, cytogenetic or molecular analysis was not performed; therefore we cannot rule out the possibility that leukemic cells, in spite of the morphological features, lacked the RARα-PML gene recombination that seems to be required for clinical response to ATRA.

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EFFECT OF ALL-TRANSRETINOIC ACID ON THE PROCOAGULANT ACTIVITY OF PROMYELOCYTIC BLAST CELLS IN CULTURE

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Blasts from 8 patients with M3 leukemia were cultured in RPMI-FCS 15% in the presence or absence of all-transretinoic acid (ATRA) 10^{-6} M. Blasts from marrow (day 0) and from cultures (days +3, +6, +9) were washed, resuspended in PBS ($5x10^{-6}$/mL) and lysed by freezing and thawing. Cell lysate was assayed for procoagulant activity (PCA) (recalcification assay), elastase activity (ELA) (amidolytic method), tissue factor antigen (TF) (ELISA) and t-PA antigen (ELISA). PCA was expressed as arbitrary units usin a reference curve (Thrombosil). Levels of PCA and TF (mean+SEM/$1x10^{-6}$ cells) are reported in the table below.

	PCA (U)		TF (pgr)	
Day	–ATRA	+ATRA	–ATRA	+ATRA
0	10.5±5.7		107±16	
+3	20.7±9.0	5.6±1.8	120±82	29±20
+6	23.5±9.3	7.3+2.7	153±99	23±6
+9	40.2±18.5	6.7+1.7	230±175	28±8

In the marrow blasts PCA and TF were higher than the control PMNs (2.8 U e 15 pgr/10^{-6} cells, respectively) and progressively increased in the cultures without ATRA up to values 2-4 fold higher than the basal ones; in the presence of ATRA PCA and TF levels were lower than the basal ones all through the culture time. In this system PCA was factor VII-dependent and was reduced to about 45% of the initial value after incubation of the lysate with concanavalin A; incubation with iodoacetamide reduced the PCA of the lysate only of 18%. ELA and t-PA did not show any significant difference during the culture time both in the presence or in the absence of ATRA. Thus a major role of PCA in pathogenesis of M3 coagulopathy is suggested; the correction of this complication by ATRA administration seems mainly mediated by its action on the PCA.

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EFFECT OF GEMCITABINE AND GEMCITABINE PLUS ARA-C ON CELLS FROM PATIENTS AFFECTED BY CHRONIC MYELOID LEUKEMIA IN BLASTIC PHASE

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The pirimidine analogue gemcitabine (dFdC) inhibits growth of LAM cells *in vitro* and increases the ARA-C cellular uptake, thus potentiating its chemotherapeutic action. We studied the effect of dFdC on blast cells from peripheral blood and bone marrow of 5 patients affected by chronic myeloid leukemia in blastic phase (CML). Mononuclear cells were separated by 1.077 g/mL density gradient and cultured *in vitro* in the presence of dFdC (1 uM, 10 uM, 100 uM), ARA-C (5 uM) and ARA-C plus dFdC (1 and 10 uM).
After 2 days of culture the following parameters were determined: 3H-Thymidine (TdR) uptake; blasts distribution in the cell cycle phases by cytofluorimetric analysis of propidium iodide stained cells; the INT reduction ability as a marker of metabolic activity and cell growth. At the same time the peak of apoptotic cells was evaluated at the end of culture by cytofluorimetic study. The apoptosis was also confirmed by DNA extraction and electroforesis on agarose gel for demonstrating the presence of endonucleosomic digestion products (bands of 200 pb and multiples) characteristic of programmed cell death.
dFdC impairs proliferation of LMC blasts. Indeed Tdr uptake is significantly inhibited by dFdC 10 and 100 uM (50 and 75 % decrease respectively in comparison with untreated controls). Moreover the combinations with ARA-C seems to potentiate this inhibiting effect. dFdC alone and in combination with ARA-C impairs INT reduction in agreement with Tdr uptake inhibition. LMC blasts cultured with dFdC 10 uM develop apoptosis, as demonstrated both by the presence of an aploid peak at the cytofluorimetric analysis and the tipic ladder aspect at the electrophoresis. Our *in vitro* data suggest a possible employ of the new drug dFdC for the treatment of blastic phase of LMC. Further studies on the activity and farmacodynamic of dFdC are required.

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FLUDARABINE + ARA-C + G-CSF: CYTOTOXICITY AND INDUCTION OF APOPTOSIS ON ACUTE MYELOID LEUKEMIA (AML) CELLS

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The adenosine nucleoside analogue fludarabine is able to increase the phosphorylation and the cytotoxicity of cytosine-arabinoside (Ara-C) both *in vitro* and *in vivo*. The combination fludarabine + ara-C + granulocyte colony-stimulating factor (G-CSF) has proven to be a highly effective regimen in poor prognosis acute myeloid leukemias (AML) and myelodysplastic syndromes (MDS).
In this study we further investigated the effects of this drug combination. *In vitro*, on fresh AML cells from 10 patients, our results confirmed an additive cytotoxic effect displayed by fludarabine + Ara-C, as demonstrated by isobologram analysis of the data. The addition of G-CSF significantly increased the efficacy of the drug combination. These effects appeared to be related to an increased incorporation of [3H]Ara-C into cellular DNA in the presence of fludarabine + G-CSF.
Furthermore, the quantitative evaluation of programmed cell death (apoptosis) showed that fludarabine + Ara-C + G-CSF induce apoptosis to a higher degree than either compound alone. Linear regression analysis showed a positive relationship between cytotoxic effect and apoptosis.
These findings suggest that cooperative induction of apoptosis could be the potential mechanism of action of this drug combination.

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CYTOGENETIC AND CLINICO-HEMATOLOGICAL CORRELATIONS IN 38 SECONDARY ANLL AND MYELODYSPLASTIC SYNDROMES

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In a five years period 38 consecutive MDS/ANLL were studied to evaluate the frequency of chromosome abnormalities and to test their impact on clinical features including survival. An occupational contact with toxic chemicals occurred in 14 patients. Twenty-four MDS/ANLL were secondary to treatment for Hodgkin disease (6 cases), for policytemia vera (6 cases), for breast carcinoma (3 cases), for myeloma (1 case), for thrombocytemia (1 case), for ovary carcinoma (1 case), for endometrial cancer (3 cases) and for thyroid cystcarcinoma (2 cases). Twenty-three patients were classified as MDS (7 RAEB and 16 RAEB-t), fourteen as ANLL (one M1, two M2, one M3, seven M4 and three M5) and one as ALL-L2. Clonal chromosome abnormalities showed an incidence of 92%. Chromosomes 5,7,11,12,17 and 17 were the most frequently involved in structural and numerical aberrations. The presence of three or more chromosome abnormalities (complex karyotypes) was the commonest cytogenetic picture observed. Del 11q23 as sole karyotipic abnormality was detected in four patients, all diagnosed as ANLL-M4. None of them had showed a preceeding myelodysplastic phase and had been treated with topoisomerase II inhibitors (antracyclines and epipodophillotoxin derivatives) for another cancer. A complete remission (CR) lasting 51, 48 and 12 months respectively was reached in the two patients with a normal karyotype and in one patient with del 11q23. No secondary MDS/ANLL with complex karyoptypes achieved CR. Our data show that complex karyotypes are frequently detected in secondary MDS/ANLL. Chromosome 5,7 and 11 are often rearranged and therefore contain genes crucial for disease development. Normal karyotypes are associated with a favourable prognosis, del11q23 with an intermediate one and complex rearrangements suggest a short survival and therefore may address these patients to allogeneic bone marrow transplantation that seems to offer a better chance of cure.

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ANALISYS OF 19 CASES OF POORLY DIFFERENTIATED ACUTE MYELOID LEUKEMIA (AML-M0)

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We describe our experience in the identification of 19 cases of AML-M0 categorized among 200 consecutive AML cases. Leukemic cells from our cases were morphologically marked by agranular basophilic cytoplasm, finely dispersed chromatin and prominent nucleoli. In 2 cases heavily vacuolated and monocytoid-shaped blasts were also observed. Cytochemistry (MPO, SBB, ANAE, NBE, NASDCAE, AP, PAS) was negative in 14 cases, 5 cases expressing a very faint cytoplasmic positivity for NBE (not exceeding 30% of the blasts) and ANAE (not exceeding 41%) which was sodium fluoride resistant. In these 5 cases other monocytic markers (eg CD14) were not in favor of myelomonocytic differentiation. All the cases were anti-MPO positive at frequency higher than 10%.
Phenotypic analysis also revealed myeloid features with all the patients having at least one myeloid antigen (CD13, CD33, CD15), Tdt was expressed in 9 cases and CD7 in 6 cases. All cases but one were positive for CD34.
Cytogenetic analysis, performed in 16 cases, showed no adequate growth in 2 cases and no consistent abnormality in 4; among the remaining 10 cases no consistent abnormality was observed, the most common finding was trisomy 8 (2 cases) and 4 (2 cases) and aberrations of chromosome 2,3,5,7,9,12 and 21. No cases of t(9;22), Ph chromosome were observed.
Interestingly 3 out of 5 patients with faint NBE/ANAE positivity relapsed as typical M4 (1 case) or M5a (2 cases). AML-M0 patients infrequently attain remission and long survival with standard therapy.
We conclude AML-M0 represents a distinct entity with remarkably poor prognosis as compared to the other FAB subgroups.

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DETECTION BY FISH OF AN ADJUNTIVE CHROMOSOMAL ABNORMALITY IN A PATIENT WITH APL

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Acute promyelocytic leukemia (APL) represents approximaly 15% of the adult non-lymphoblastic leukemias and is associated with a specific cytogenetic abnormality: the translocation of a portion of the long arm of chromosome 17 onto the long arm of chromosome 15; t(15:17)(q22;q12-21); molecular studies have revealed DNA rearrangement for the nuclear retinoic acid receptor-α (RaR-α).
We studied an APL patient, HIV-positive, presenting with disseminated intravascular coagulation and with a bone marrow infiltration > 80% promyelocytes. Cytogenetic investigation was carried out on bone marrow using 48 h incubation culture; Q-banding was routinely performed and few metaphases were fully karyotyped.
The patient showed a 47, xy, +8, t(15:17) karyotype. To overcome the difficulty to obtain metaphases suitable for the detection of the specific cytogenetic aberration, fluorescent *in situ* technique (FISH) can be an important tool for the study of such genetic alteration. A human chromosome-8 specific painting probe was used. FISH demostrated the abnormal karyotype in 100 of 100 interphase cells and in 15 of 15 metaphases analyzed, in comparison to literature data in which +8 abnormality was present as a part of mosaicism.
After induction therapy with retinoic acid (70 mg/die) and idarubicin the patient obtained a morphological and cytogenetic complete remission; t(15:17) and trisomy 8 were not detected neither with routinely cytogenetic proceeding neither with FISH. These data confirm FISH technique advantages: it is fast and relatively easy, large numbers of interphase cells can be analyzed, metaphases of a poor quality which do not allow proper banding can be evaluated and can be useful as an adjunct to conventional cytogenetic analyses in the detection of chromosome aberrations and abnormalities as this case has showed.

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MOLECULAR HETEROGENEITY OF HYPEREOSINOPHILIC SYNDROME

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The hypereosinophilic syndrome (HES) is a disorder characterized by persistent peripheral blood eosinophilia of unknown origin associated with eosinophilic infiltration of tissues. The similarity between HES and other myeloproliferative syndromes has been already emphasized in the past. However, the evidence to support a clonal proliferation of eosinophils has been sparse and only based on the detection of chromosomal abnormalities in bone marrow cells of those patients with the clinically aggressive variants of HES, otherwise known as eosinophilic leukemia. We describe two patients presenting with clinical and hematologic features consistent with HES in which hypereosinophilia represents a proliferation of a cell clone, as shown by the methylation status of the X-linked phosphoglycerate kinase (PGK) gene.
Major laboratory findings included leukocytosis ranging from 20000 cells/mm^3 to 34000 cells/mm^3 with 50-70% eosinophils and a slight polyclonal increase in serum levels of IgE. The bone marrow aspirate revealed increased cellularity with eosinophilic hyperplasia. The morphological and immunocytochemical properties (PAS and naphtol-ASD-chloroacetate-esterase) of the peripheral blood and bone marrow eosinophils were consistent with normal non leukemic cells. Conventional cytogenetic analysis showed only a normal karyotype. A biopsy of the median nerve in one patient and of the muscle as well as of the ulnar nerve in the other one demonstrated a typical eosinophilic infiltration. A clonal analysis was performed on the DNA extracted from the patients' granulocytes collected at diagnosis using a PGK probe. With BstXI digestion, two fragments of 0.9 kb and 1.8 kb were detected. The 1.8 kb fragment disappeared almost completely with subsequent digestion with HpaII in both cases. This pattern of methylation of the gene, which was consistent with clonal hematopoiesis, was also found at recurrence of the disease. Here we document that in two patients which met all the hematologic and clinical criteria of HES, blood hypereosinophilia may be sustained by a myeloproliferative process which is clonal although probably not fully malignant, as testified by the favourable clinical course and the great susceptibility to steroid treatment. The pathogenetic mechanisms underlying HES are still obscure. A possible involvement of T lymphocytes secreting an eosinophil differentiation factor has been suggested, together with the eventual occurrence of a clonal T-cell population in HES. We are still investigating this possibility in a third patient affected with a B cell lymphoma associated with hypereosinophilia showing a small clonal population of CD3-CD4+cells which might be directly linked to the production of cytokines responsible for the hypereosinophilia. We think that careful documentation of the molecular features of future HES patients is needed to confirm the clonal nature of the disease and to show if HES may constitute a distinct clinicopathologic subgroup within the myeloproliferative disorders. On the other side HES patients should be carefully examined for an undelying proliferation of clonal T cells. Therefore, a challenge for future research is to elucidate the heterogeneous nature of HES and, given the rarity of this entity, we propose that such investigations should be achieved by a multi-institutional Italian study.

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STUDY OF LINEAGE INVOLVEMENT BY NUMERICAL CHROMOSOME ABERRATIONS IN HEMOPOIETIC NEOPLASMS: A CYTOGENETIC AND INTERPHASE CYTOGENETIC APPROACH

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Because conventional chromosome analysis does not allow for the recognition of the cytological type of metaphase cells, the role of fluorescent *in situ* hybridization (FISH) as a mean of assessing lineage involvement by numerical chromosome aberrations in hemopoietic neoplasms was studied.
Three different approaches were employed in the following patients:
1. FISH on enriched cell fractions obtained by separation over a density gradient of Percoll in two patients with acute myelomonocytic leukemia (AML-M4) presenting with trilineage myelodysplasia (TMDS) and showing trisomy 11 at conventional chromosome analysis;
2. FISH on previously immunolabelled interphase cells in two patients with MDS and trisomy 8 and in two cases of chronic lymphoproliferative disorders with trisomy 12;
3. FISH on the progeny of single hemopoietic colonies grown in semisolid media from two patients with MDS evolving into AML carrying trisomy 8 as the primary chromosome change.
Our data documented consistent involvement by +11 of an erythroblast-enriched cell fraction and blast enriched cell fraction in both patients with AML-M4 and TMDS, whereas heterogeneity of lineage involvement by +8 was documented in MDS evolving into AML both by combined FISH/immunophenotyping and by FISH on single CFU-GM and BFU-E. A higher trisomic/disomic cell ratio within each cell lineage was found when comparing data obtained by FISH on immunolabelled cells and FISH on hemopoietic colonies, suggesting that preferential *in vitro* growth of disomic progenitor cells may have occurred in these patients.
In addition, trisomy 12 was shown to be restricted to CD19+ and CD11c+ cells in two cases with B-cell chronic lymphocytic leukemia and hairy cell leukemia, respectively.
It is concluded that different approaches employing FISH may be of value for the study lineage involvement by numerical aberrations in hemopoietic neoplasms

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MOLECULAR ANALYSIS OF ALL-1 GENE IN HEMATOLOGICAL MALIGNANCIES OF THE ADULT

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11q23 region has been reported to be frequently involved in a number of non-random chromosomal abnormalities, mostly in acute leukemias, both lymphoid and myeloid, and more rarely in other hematologic malignancies as non-Hodgkin lymphomas. Recently a genomic locus, variously called ALL-1, MLL or HRX, has been cloned and characterized, and it has been demonstrated to be involved in the vast majority of cases bearing 11q23 abnormalities. Nevertheless, the possible occurrence of genetic lesions involving the ALL-1 gene, but not detectable by the cytogenetic analysis, has not yet been comprehensively investigated at the molecular level in hematologic tumors others than acute leukemias. Prompted by these considerations, we investigated the frequency of the molecular involvement of ALL-1 gene in a large panel representative of the different hematological malignancies, independently from the availability of cytogenetic data.
Taking advantage of a 0.7 kb cDNA probe spanning the 11q23 breakpoint region of ALL-1, we performed Southern Blot analysis on 404 cases of hematologic malignancies of the adult, represented by 44 chronic lymphocytic leukemias (CLL), 47 mature B-cell chronic lymphocytosis (BCL), 88 low, intermediate and high grade non-Hodgkin lymphomas (LNH), 64 multiple myelomas (MM), 32 acute lymphoblastic leukemias (ALL), 110 de novo acute myeloid leukemias (AML), 16 AML derived from myelodisplastic (MDS) and 3 AML derived from myeloprolypherative syndromes (MPS). We detected rearrangements of the ALL-1 gene in 4 cases of ALL (4/32; ≈13%) and in 13 cases of AML (13/129; 10%), whereas no structural alterations of this gene were found in all the remaining types of neoplasia. In 4 rearranged cases (3 AML and 1 ALL) for which no cytogenetic data were available, we evidenced a ALL-1/AF-4 fusion product, the molecular counterpart of t(4;11), by RT-PCR analysis. In the only case harbouring a known t(4;11), RT-PCR analysis confirmed the karyotype findings.
In conclusion, our study confirmed the frequency and the specific involvement of ALL-1 gene in ALL and AML, and demonstrated by molecular analysis the absence of structural lesions in a large representative panel of lymphoproliferative diseases of the adult.

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INTERSTITIAL DELETION OF BAND q14 OF CHROMOSOME 13 LOCUS OF RB1 GENE IN NON-HODGKIN LYMPHOMA

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The product of retinoblastoma gene (RB1), localized to q14 band of chromosome 13, results to be a cell cycle regulator with an oncosuppressor effect.
The inactivation or the complete loss of its function represents one of the mutation, necessary even if not sufficient, to develop different types of tumors. Heterozigosity for RB1 gene, constitutional or not, that can arise from point mutations, insertions, microdeletions or chromosome abnormalities in 13q14, confers a dominant predisposition to the development of tumors, such as retinoblastoma, sarcomas and other epithelial tumors.
Interstitial deletions of 13q14 region are a cytogenetic marker indicative of the involvement of RB1 gene in the cancerogenesis of a neoplastic cell population.
In leukemias, molecular abnormalities of RB1 have been observed in 10-30% of acute diseases and in blastic crisis of chronic myeloid leukemias Ph+ and Ph–.
In the indolent phase of leukemias and lymphomas, mutations of RB1 oncosuppressor gene are rare and their effect has been associated to the stage of progression.
In chronic lymphocytic leukemias rearrangements of 13q region are the most frequent alterations (15%) after trisomy 12 and represent a positive prognostic factor, compared with the other alterations.
In non-Hodgkin lymphomas (NHL), cytogenetic data concerning 13q14 deletion and molecular alteration of RB1 oncosuppressor gene are still quite poor.
We report the finding of the 13q14 deletion in a NHL (centrocytic-centroblastic, intermediate grade), at diagnosis, with a previous recent story of ovary and colon carcinoma, whose karyotype was 46,XX,9q+, del(13)(q12q14),t(14;18) in the lymph node cells, and an immunoblastic lymphoma (high grade of malignancy), at diagnosis, with karyotype 51-54,XXY,+5,+7,+9,+12,+14,+21, del(13)(q12q14).
The analysis by fluorescent *in situ* hybridization (FISH) with a specific probe for the whole chromosome 13 (painting probe) did not show, in both neoplastic cell populations, any segment of chromosome 3 translocated to the other chromosomes, thus confirming the deletion of 13q14 band and RB1 oncosuppressor gene.

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MOLECULAR ANALYSIS OF CUTANEOUS B AND T CELL LYMPHOMAS

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Cutaneous lymphomas represent a heterogeneous group of lymphoid malignancies in which the molecular pathogenetic mechanisms are still poorly understood. Primitive cutaneous T-cell lymphomas (CTCLs) are more frequent and better studied, allowing the definition of new pathological entities. Recently, primitive cutaneous B-cell lymphomas (CBCLs) have been characterized, and a possible origin from the follicular centre cell has been proposed for the majority of them. The disruption of cellular growth and differentiation control, due to genetic lesions affecting oncogenes and tumor suppressor genes, have been demonstrated to underlie the pathogenesis of many different human cancers. In the context of lymphoid malignancies best understood examples are represented by c-myc rearrangement in Burkitt's lymphomas, bcl-2 rearrangement in follicular lymphomas, bcl-1 rearrangement in intermediate lymphomas, ras mutation in MM and ALL, p53 inactivation in Burkitt's lymphomas, MM, B-CLL and in the diffuse/advanced cases of follicular lymphomas. Prompted by these considerations, with the aim to contribute to a better definition of the molecular pathogenesis of this subset of non-Hodgkin's lymphomas, we have investigated the involvement of several proto-oncogenes (namely c-myc, tal-1, bcl-1, bcl-2, bcl-3, bcl-6 and NFKB2/lyt-10) and of the p53 tumor suppressor gene in a representative panel of cutaneous lymphomas, including 23 cases of cutaneous B-cell lymphomas (10 primitive centroblastic/centrocytic lymphomas; 4 centroblastic/centrocytic follicular/diffuse lymphomas; 9 centroblastic lymphomas) and 36 cases of cutaneous T-cell lymphomas (20 mycosis fungoides; 5 CD8+ cutaneous lymphomas; 8 pleomorphic Ki1+ lymphomas and 3 anaplastic large cell lymphomas Ki1+). Within CBCLs we detected bcl-1 locus rearrangement in 1 case, bcl-2 rearrangement in 2 cases, NFKB2/lyt-10 gene rearrangement in 2 cases, bcl-6 rearrangement in 1 case, whereas no rearrangements of c-myc and of bcl-3 genes were detected. Among CTCLs, we detected rearrangements of the NFKB2/lyt-10 gene in 2 cases and of tal-1 gene in 3 cases. Analysis of p53 gene in all the cases of CBCLs and of CTCLs by PCR-SSCP-direct sequencing approach, showed mutation in one case of mycosis fungoides in tumoral stage, occurring at codon 163 (TAR→CAC; Tyr→Asp). Our data show that genetic lesions involving different oncogenes and tumor suppressor genes are involved in a non-specific way in the pathogenesis of primary CBCLs and CTCLs in a significant, even if limited, proportion of cases (11 out of 59 primary cutaneous lymphomas; ≈18%). Furthermore, the different molecular characteristics of these malignancies in comparison to their immunocytomorphological counterpart originating from the lymph node, suggests that cutaneous lymphomas may represent a distinct biological entity.

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CD44 GENE SPLICE ALTERATIONS IN NON-HODGKIN'S LYMPHOMAS

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CD44 is a transmembrane group of glycoproteins involved in the interactions between cells and extracellular matrix and supposed to play an important role in lymphocytes recirculation. CD44 gene may generate a large number of different CD44 isoforms by alternative splicing of at least 10 *variant* exons. The smallest splice CD44 gene product is a 85 Kd protein, called *standard form*, that is the major isoform expressed on lymphocytes. Activations of T-lymphocytes increase CD44 expression and may generate some CD44 variant forms. Moreover CD44 variant forms have been found to be over-expressed in a variety of human cancers and tumor cell lines. Some of these variant forms have been shown to be causally involved in tumor metastasis formation in experimental models. The similarity between tumor spread and lymphocytes recirculation and the supposed role of CD44 in both processes raise the question whether CD44 splice variants might have a role in migration of malignant lymphocytes.
Therefore we decided to investigate CD44 splice variants in RNAs extracted from normal human peripheral lymphocytes and non-Hodgkin's lymphoma (NHL) tissues. Ten total RNAs extracted from NHL lymph node tissues of different hystological subtypes, from 4 biopsies derived from patients affected with inflammatory diseases and from PBMCs of two healthy subjects were studied by RT-PCR using as primers two oligonucleotides able to amplify across the site of insertion of CD44 variant exons. After 35 cycles of PCR the amplified products, representative of the different forms of CD44 mRNAs present, were analyzed by Southern blot and hybridized with an oligonucleotide capable to recognize all CD44 splice products and with an oligoprobe able to recognize CD44 Hofmann's domain 3 (D3). A fragment of 482 bp, indicative of the standard form, was present, at almost the same intensity, in all the samples. In PBMCs were also present 3 further forms, containing D3 domain at a very low level. The same pattern was present in the 4 lymphadenopathy samples. Four out of 6 aggressive and 1 out of 4 low grade NHLs expressed variant forms with differencies in number, size, and intensity of the bands obtained from PBMCs and lymphadenopathy samples.
These results indicate the presence of some CD44 splice variants in NHLs, expecially in high-grade sub-types, suggesting a possible role in promoting dissemination of lymphoma cells, probably via alterations of the interactions with high endotelial venules.

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MOLECULAR CYTOGENETIC ANALYSIS IN A PATIENT WITH MULTIPLE MYELOMA AND POLYCYTHEMIA VERA

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A 64-year-old man was diagnosed as having polycythemia vera and smoldering IgGK myeloma in 1991. At diagnosis, osteolitic lesions were not detected and at sternal puncture 5% of plasmacells in a hypercellular marrow were observed. The karyotype was normal. In 1993, the patient developed a full-blown myeloma with multiple bone lesions. At that time, karyotype was normal in 95% of the metaphases, whereas 5 out of 120 cells showed a complex karyotype with trisomy for chromosomes 3,5,6,9,15,18 and a marker apparently derived from chromosome 7. As the patient presented two distinct hematological disorders, we used a FISH analysis in order to clarify:
1) the percentage of chromosomally abnormal cells;
2) the involvement of 7q in the derivative chromosome;
3) the hematological lineage carrying the abnormal karyotype.
First, we hybridized a centromeric probe for chromosome 6 on the cytogenetic pellet; three signals were observed in 12.6% of the cells. We afterward used dual color fluorescence to define whether the marker really belonged to chromosome 7. The biotinilated centromeric probe for chromosome 6 was mixed with a digoxigenin-labeled probe for chromosome 7. Three yellow and three red signals were observed in 9.4% of the cells: this allowed us to characterize the marker chromosome as a derivative of 7q. We performed a third FISH experiment hybridizing a centromeric probe for chromosome 6 directly on bone marrow smears, previously stained with May-Grünwald Giemsa. Three signals were observed only in the plasmacells; all myeloid cells had two yellow spots.
In this case we demonstrated that FISH may be used both for detection of chromosomal abnormality in cells with a low mitotic index and for identification of aneuploid cells directly on bone marrow smears.

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RARE TRISOMIES IN MYELODYSPLASTIC SYNDROMES: CYTOGENETIC AND FISH STUDIES

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Trisomy 8 and monosomy 7 are the most frequent numerical changes in myelodysplastic syndromes (MDS). More recently trisomy 14 has been described in malignant disorders with hematological features between myelodysplastic syndromes and Ph-negative chronic myeloid leukemia. We here report on two rare trisomies, i.e., trisomy 6 and trisomy 11, as isolated karyotypic changes in two patients with MDS, providing new insights to the significance of these aberrations in myeloid disorders.
A 50-year-old woman presented with pancytopenia and a bone marrow consistent with aplastic anemia. Detection of trisomy 6 in 40% of bone marrow karyotypes allowed us to diagnose a myelodysplastic syndrome. As far as we know eight cases of MDS with trisomy 6 have been reported until now (including the present case). Aplastic bone marrow is the common cellular feature in all these cases.
A 84-year-old man had a refractory anemia with excess of blasts (RAEB) and 78% of bone marrow karyotypes with trisomy 11. The disorder rapidly evolved to RAEB in trasformation, while the karyotype unchanged. Trisomy 11 has been reported only in myeloid disorders, namely in acute non lymphocytic leukemia. Sporadic cases were classified among MDS.
Trisomic clones in both these cases were further characterized at diagnosis and during follow-up by FISH on interphase nuclei from chromosome preparations as well as from peripheral blood and bone marrow smears. The clonal numerical change, even in a very small percentage of cells, was shown over the course of MDS, despite of treatments. The presence of trisomic polymorphonucleated cells in both situations suggest that either trisomy 6 or trisomy 11 are compatible with persistence of myeloid differentiation. Trisomy 6 was strongly related to dysplastic erytroid cells in the bone marrow supporting the classification of trisomy 6 associated disorders among myelodysplastic syndromes.

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FINGERPRINTS OF AMPLIFIED THIRD-COMPLEMENTARY-DETERMINING-REGION (CDR-III) FOR MONITORING THE MINIMAL DISEASE IN B-CELL LINEAGE ACUTE LEUKEMIAS

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Approximately 90% of hematopoietic malignancies of the B-cell lineage have a clonal immunoglobulin heavy chain gene rearrangements due to the recombination of variable, diversity, and joining regions of the heavy-chain gene segments during B-cell commitment. These rearrangements result in a region called complementary-determining region III (CDR-III). This region, which encompasses the diversity region of the heavy-chain segment, because of extensive somatic mutations, provides a DNA-encoded signature specific for each B-cell clone.
We have developed a simple polymerase chain reaction (PCR) based method for detecting IgH gene rearrangement, using the CDRIII region. The size heterogeneity resulting from independent IgH rearrangement events and the high resolution power after electrophoresis and silver staining of the PAGE gels can be used to generate a *fingerprint* representing either the spectrum of B cell clonality in complex populations of B lymphocytes or the partially genomic configuration of the VH-N-DH-N-JH region.
We illustrate the application of the method in assessing the spectrum of B cell clonality occurring in a series of 38 acute lymphoblastic leukemia. We explore the application of the technique in tracking minimal residual disease and for monitoring clonal evolution in acute lymphoblastic leukemia. In two cases the amplified have been sequenced in order to use the CDRIII regions to generate diagnostic probes that hybridized only to the amplified CDR-III of leukemic cells from which the sequences were derived, providing a specific and diagnostic marker for each B cell clone.

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MISLEADING CYTOGENETIC SUGGESTION OF LYMPHOID RELAPSE IN DONOR CELLS AFTER ALLOGENEIC BONE MARROW TRANSPLANTATION (BMT) CORRECTED BY FISH ANALYSIS IN A PATIENT WITH PH' NEGATIVE CHRONIC MYELOGENOUS LEUKEMIA (CML)

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A 30-year-old female was diagnosed as having bcr/abl positive (b2a2, p21O), Ph' negative CML on October, 1992. The patient received effective therapy with hydroxyurea followed by α-interferon until April, 1993, when she successfully underwent allogeneic BMT from her matched male sibling. On April, 1994 the patient showed a hematological relapse presenting with morphological and immunological features of a common- acute lymphoblastic leukemia. Blood group still was that of the donor. In this phase, conventional cytogenetics carried out on bone marrow (blast cells > 95%) demonstrated exclusively the presence of normal male (46,XY) metaphases, thus suggesting the possibility of a relapse in donor cells. However, FISH analysis, performed on the same preparation using α-satellite probe DXZ1 (Oncor), specific for chromosome X, evidenced the female origin of leukemic cells, showing two signals in about 9O% of the interphase nuclei and one signal in the remaining 10% (male donor cells), including all observed metaphases. After treatment with vincristine, doxorubicin and prednisone, the patient achieved complete remission during which bone marrow combined analysis by cytogenetics and FISH showed 1OO% of normal male cells. Serial molecular studies are in progress. This report represents a further demonstration of the usefulness of FISH in the evaluation of particular situations in which cytogenetics alone may not provide conclusive results.

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PREVENTION OF APOPTOSIS IN NEUTROPHIL GRANULOCYTES: ROLE OF TYROSINE-KINASES

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In vivo, senescent neutrophil granulocytes (PMN) are supposed to die by apoptosis. PMN derived apoptotic bodies are then removed by macrophages. *In vitro*, apoptosis of PMN can be induced by culture in the absence of appropriate growth factors or cytokines.
Since in growth factor dependent cell lines activation of cytoplasmic tyrosine-kinases is an early event following stimulation with growth factors, we evaluated if this is the case also in PMN and if activation of tyrosine-kinases is necessary for prevention of apoptosis in these terminally differentiated cells. PMN were isolated from the peripheral blood of normal subjects and were cultured in the presence or absence of hemopoietic growth factors (GM-CSF and G-CSF), lipopolysaccharide, inhibitors of tyrosine-kinases and other agents which interfere with different systems of signal transduction from growth factor receptors. At 24-48 hours the number of viable cells, morphology and presence of DNA fragmentation were evaluated.
Our results confirm that growth factors and lipopolysaccharide protect PMN from apoptosis (30% of porphologically apoptotic cells following culture for 24 h without growth factors compared with 4-8% with growth factors). Inhibitors of tyrosine-kinases are only mild inducers of apoptosis (8-12% of apoptotic cells at 24 h). This is in contrast with the situation in growth factor dependent cell lines where tyrosine-kinase inhibitors induce a higher degree of apoptosis than culture without of growth factors. In cell lines apoptosis induced by both growth factor deprivation and inhibitors of tyrosine-kinases is associated with a reduction in the fraction of cells in the S and G2/M phases of the cell cycle, and the tyrosine phosphatase inhibitor Na-orthovanadate prevents both kinds of apoptosis.
This suggests that the signal transduction systems involved in prevention of apoptosis by growth factors are, at least in part, different in PMN and cell lines. Tyrosine kinases appear to be important for prevention of apoptosis mainly in cells during the S and G2/M phases of the cell cycle, and less important in G0 cells such as PMN.

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CELL LOSS BY APOPTOTIC MECHANISMS INDUCED IN PERIPHERAL BLOOD LYMPHOCYTES STIMULATED WITH INTERLEUKIN-2 AT DIFFERENT DOSES

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Apoptosis represents a mechanism of cell loss that is programmed during the turnover of tissues in normal, pathological and stimulated conditions. This phenomenon is characterized by a formation of residual apoptotic bodies that are internalized by cells with phagocytic properties. It is well known that high doses of recombinant interleukin-2 (rIL-2) can induce cytotoxic effects *in vivo*.
In order to investigate the relationship between high doses of recombinant interleukin-2 (rIL-2) and the appearance of apoptotic cells in PBL stimulated *in vitro* with increasing doses of rIL-2 (1, 10, 50, 100, 500, 1000 IU/mL for 7 and 14 days) an ultrastructural study has been developed. These results were compared with those obtained *in vivo* in peripheral blood cells from treated subjects 24 and 48 hours after rIL-2 subministration and after growing of these cells for 7 and 14 days *in vitro* with 1000 IU/mL rIL-2.
The data revealed that low doses of rIL-2 (up to 50 IU/mL) don't induce apoptosis, while a very strong apoptotic pattern with a presence of different stages of the ultrastructural morphology, was observed starting from 100 IU/mL. The lymphocytes showed an intense degenerative activity with different stages of apoptosis till the cytoplasmic lysis.

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ENHANCED SUSCEPTIBILITY TO APOPTOSIS IN T CELLS OF MULTIPLE MYELOMA

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Activated (HLA-DR$^+$) T cells have been reported in the peripheral blood of multiple myeloma (MM) patients. Though evidence for a T cell-based tumor-host interaction, these cells are negatively correlated with disease status and prognosis and display defective reponses to a number of stimuli.
The aim of this study was to shed some light on the molecular mechanisms underlying their functional impairment. The expression of Fas (CD95) and bcl-2 antigens as well as the susceptibility to spontaneous and secondary apoptosis were evaluated in 15 MGUS, 30 MM, and 18 age-matched controls. Fas was significantly increased in MGUS (37±16%) and MM (63±27) as compared to normal controls (24±16%), while bcl-2 expression was significantly higher in MGUS (94±6%) and lower in MM (67±26%) than in normal controls (82±12%). Spontaneous apoptosis (following incubation in medium + 1% FCS) and secondary apoptosis (following exposure to apoptotic agents such as methylprednisolone and anti-Fas mAb) were assessed in purified T cells with a triple procedure and confirmed with DNA fragmentation analysis. Both spontaneous and secondary apoptosis were significantly higher in MM than the controls.
These data indicate a failure of apoptosis regulation in MM T cells which may partially account for the poor performances of the T cell compartment in these patients.

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DETECTION OF ACTIVATED EOSINOPHILS BY FLOW CYTOMETRY (FOG METHOD)

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Laboratory procedures classically employed to isolate eosinophils had several disadvantages such as need for large volumes of blood, contamination with neutrophils and surface receptor modulation. A recently developed technique, the FOG method, allows both the cytofluorimetric identification of eosinophils from unseparated peripehral blood (PB) based on their light scattering properties and the simultaneous detection of surface and intracellular antigens (respectively, CD4, CD9, CD11a, CD11b, CD18, CD23, CD25, CD54, HLA-DR and EG1, EG2, Estrogen Receptor). The cell membrane permeabilization is achieved by treating paraformaldehyde-fixed leukocytes with n-octyl-β-D-glucopyranoside (OG); such procedure is reported not to affect antibodies already bound to cell membranes (*G. Hallden, 1993*).
Eosinophils can be recognized as a well separated population with a high surface expression of CD9 and preserved high scatter signal. A possible explanation of such stability may be the crystalloid structure of eosinophil major basic protein (MBP).
We examined the immunophenotype of PB eosinophils from a group of 10 normal blood donors and from a pt submitted to IL-2 immunotherapy for acute myelogenous leukemia (AML). Such treatment is known to determine eosinophilia, probably mediated through IL-5 secretion by activated T-lymphocytes.
Our results demonstrate, with respect to the control group, an increased expression of cell surface (IL-2 receptor α-chain or CD25) and intracellular (EG1-antiECP- and EG2-antiECP/EPX) activation markers and a decrease of eosinophil complexity/granularity as assessed by side scatter signals.

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EOSINOPHIL CATIONIC PROTEINS ENHANCE TNF AND HYDROGEN PEROXIDE RELEASE BY HUMAN MONOCYTE-DERIVED MACROPHAGES

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The presence of a florid eosinophil infiltrate has been shown to positively correlate with a favorable prognosis in a variety of human tumors and to be accompanied by inhibition of growth or eradication of experimentally induced tumors in animal models. The eosinophil infiltrate is invariably accompanied by the presence of numerous macrophages.
Macrophages are known to play a fundamental role in host defence against tumors. Hence, the possibility has been considered that a collaboration may exist between these two cell types in the control of tumor growth.
Eosinophil granulocytes contain in their secondary granules large amounts of highly cationic proteins that have been shown to affect the functions of a variety of cell types. In the present study we examined the effect of three of these proteins, i.e. eosinophil cationic protein (ECP), eosinophil-derived neurotoxin (EDN), and eosinophil peroxidase (EPO), on TNF production and hydrogen peroxide release by human monocyte-derived macrophages. After incubation with ECP, EDN and EPO macrophages produced large amounts of TNF and displayed an enhanced PMA-triggered hydrogen peroxide release with the following order of potency: ECP> EDN>> EPO. These effects were accompanied by morphologic changes leading the large, round macrophages of the control cultures to acquire an elongated, pear-shaped or spindle-shaped appearance after treatment with EPO and a fibroblast or star-like appearance after treatment with ECP and EDN. EPO but not ECP and EDN caused also an increase in cell protein content. Other cationic proteins such as lysozyme and MPO, the peroxidase of neutrophils that has a catalytic activity similar to that of EPO, were ineffective.
The finding that eosinophil-derived secretory proteins stimulate the cytotoxic weaponry of macrophages supports the hypothesis for a role of an eosinophil-macrophage cooperation in the control of tumor growth.

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EVUALUATION OF THE EFFECT OF THYMULIN ON NORMAL T LYMPHOID PRECURSORS

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Thymulin is a nonapeptide hormone isolated from the thymus gland. Its major actions have been shown to be on T-cells and their immature precursors.
In this study we investigated the effect of thymulin on the growth of T cell precursors from the peripheral blood mononuclear cells (PBMC, $CD2^+$ $HLA\text{-}DR^-$) of normal volunteers by using a T cell colony assay (*Izaguirre et al. 1981, Consolini et al. 1985*). Thymulin was tested at various concentrations (from 5 to 20 l/mL) in a dose-response curve.
Thymulin alone did not induce colony formation. In addition to PHA-leukocyte conditioned medium (PHA-LCM), it was able, at the optimal concetration of 5 l/mL, to significantly enhance either ^{3}H-Tdr uptake ($p< 0.05$) or colony growth ($p< 0.05$) of $CD2^+$ $HLA\text{-}DR^-$ population. In addition, thymulin at the concentration of 1 ng/L, was tested *in vitro* for its effects on the release of IL2, IL6, GM-CSF from normal PBMC stimulated with PHA and cultured for 72 hours.
Surnatants subsequently assayed for cytokine activities resulted in an increase of GM-CSF in PHA-LCM-thymulin induced surnatant compared to that one obtained from PHA-LCM stimulated lymphocytes. As thymulin enhances IL2 receptor induction on T lymphocytes, the same IL2 level found either in PHA-LCM-thymulin or PHA-LCM induced surnatants could reflect the IL2 consumption from T activated cells, suggesting a previously reported indirect effect of thymus on myelopoiesis. On the contrary, thymulin inhibited IL6 release from PHA-LCM-thymulin stimulated lymphocytes compared to PHA-LCM stimulated lymphocytes.
Our preliminary results stress on the role played of the thymus in the immune control of hematopoiesis, by stimulating the proliferation and hematopoietic growth factors production by T lymphocytes.

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MORPHOFUNCTIONAL CHARACTERISTICS OF LAK CELLS

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The morphofunctional features of lymphokine activated killer (LAK) cells obtained from peripheral blood lymphocytes (PBL) cultured in the presence of recombinant interleukin-2 (rIL-2) at low doses (100 pg/mL) for 10 days were studied. The cytotoxic activity of these cells was evaluated against normal cells (fibroblasts) and against different transformed cell lines (Daudi, Molt4, K562, Chang, HeLa). Moreover, the cytochemical characterization of LAK cells was performed using a panel of cytochemical reactions: lysosomal hydrolitic activities as acid phosphatase, β-glucuronidase, α-naftil butirate esterase, dihydrofolate reductase (DHFR), dypeptidil-aminopeptidase (DAP IV) and serine esterase.
The morphological and cytochemical study was performed on cells previously marked with anti-CD4 and anti-CD8 beads allowing in this way the simultaneous immunological identification of the cells. The behaviour of these IL-2 responding cells was compared with that of peripheral blood lymphocytes obtained from patients treated with IL-2 (Atzpodien protocol).
Our data supported the hypothesys that LAK obtained in our experimental condition *in vitro*, belong to CD4+ subset, showed *hand mirror shape* (HMS) and the lack of lysosomes.
IL-2 *in vivo* induces the differentiation of HMS cells in peripheral blood with the same morphofunctional characteristics.

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EFFECT OF ILOPROST THERAPY ON LEUKOCYTES INTEGRIN

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Iloprost, a stable prostacyclin analogue, is known to have beneficial effects on the disturbed microcirculation. Particularly this drug shows an important role on the reducing leukocytes adhesion to damaged vascular endothelium.
We have investigated the effect of iloprost on phagocytes' function and their adhesion molecules of patients suffering from vasculitis and progressive systemic sclerosis (PSS).
We studied 10 patients as control that received infusion of physiological solution for 6 hours and 20 patients, 8 suffering from peripheral arterial occlusive disease Fontaine's stage III and IV that received infusion of iloprost for 6h/day for 28 days and 12 suffering from PSS that received infusion for 6h/day for 14 days. The range of iloprost infusion was 0.5-2.0 ng/kg/min. We collected whole blood of each patients the first day of therapy pre-treatment and post-treatment. We evaluated a superoxide production (O_2^-) of PMN and expression on leukocytes of integrin $\alpha_L\beta_2$ (CD11a/CD18), $\alpha_M\beta_2$ (CD11b/CD18) and $\alpha_X\beta2$ (CD11c/CD18) by flow cytometric analysis and our results are express as mean channel fluorescence intensity. Iloprost therapy induced an evident reduction in the expression of $\alpha_M\beta_2$. There was a significative correlation between the expression of $\alpha_M\beta_2$ on PMN before-therapy and after-therapy respectively 80.6±20.4 MFI vs. 40.24±7.7 MFI, $p<0.01$ and the expression of aMb2 on monocytes pre-therapy and post-therapy, respectively, 86.85±19.29 MFI vs. 58.11±11.2 MFI, $p<0.01$. In our results we observe the same for $\alpha_X\beta_2$, while the $\alpha_L\beta_2$ we don't show different before and after drug infusion.
The O_2^- production in basal condition was significantly decreased between pre-therapy and post-therapy respectively, 75.2±23.1 MFI vs. 44.3±12.1 MFI, $p<0.01$. There was no correlation between pre-therapy and post-therapy O_2^- production when we stimulated PMN with PMA 10^{-7}M and fMLP 10^{-8}M.
All these changes induced by iloprost are important for modulating the interaction between leukocytes and endothelial cells. The effect of prostacyclin analog reduce the toxicity of leukocytes in endothelial injury. The reduced number of $\alpha_M\beta_2$ decrease leukocytes adherence to endothelial cells. Finally iloprost reduced the capacity of leukocytes to release O_2^- and modified $\alpha_M\beta_2$ up-regulation limiting the endothelial vascular injury.

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LECTINS AS A TOOL TO STUDY ERYTHROCYTE MEMBRANE GLYCOSYLATION DEFECTS

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Lectins are proteins of nonimmune origin characterized by their high specific affinity to carbohydrates in glycoproteins and glycolipids. They are useful for a variety of purposes such as functional and morphological studies of normal and pathological cells, fractionation of cells exposing lectin receptors, and isolation and structural studies of glycoproteins.
Here we report about the use of lectins as a tool to study erythrocyte membrane glycosylation in type II congenital dyserythropoietic anemia (CDA).
Type II CDA is a rare disorder characterized by refractory anemia and ineffective erythropoiesis. Type II CDA results from an inherited abnormality of the enzyme N-acetylglucosaminil-transferase II, required for the normal synthesis of the carbohydrate moieties of erythrocyte membrane; as a result of the defect, glycosylation of protein 3, protein 4.5, and glycophorin A is impaired.
Erythrocyte protein 3, the anion exchange protein, is the major component of red cell membrane, accounting for approximately 25% of total membrane protein; it is a highly glycosylated 95 kDa protein that exists in the red cell membrane as a mixture of homodimers and homotetramers.
We investigated for protein 3 glycosylation abnormalities in three unrelated patients affected with type II CDA, by means of a lectin/anti-lectin system. Briefly, red cell proteins were separated by polyacrylamide gel electrophoresis, and electroblotted onto nitrocellulose membrane. The membrane was then incubated with *Phaseolus vulgaris* erythroagglutinin (PHA-E), a lectin which specifically binds to N-acetyl-D-galactosamine on protein 3, and subsequently in peroxidase labeled anti PHA-E antibody. 4-chloro-1-naphthol/H_2O_2 was sed as chromogenic substrate. The immunostaining revealed a protein 3 heterogeneous glycosylation defect in all three CDA patients, as compared with normal controls.
We suggest that different lectins could be used to study glycosylation of different red cell membrane proteins.

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IDIOPATHIC THROMBOCYTOPENIC PURPURA: A NEW THERAPEUTIC APPROACH

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In the pathogenesis of idiophatic thrombocytopenic purpura (ITP) small and soluble immunocomplexes (SMALL CIC) may play a primary role. They are composed by PDIg (platelet directed Ig or Ig molecules against platelets), that interfere in regulation mechanism of immunologic response at following levels: limiting clearance of monocytes/macrophages and IL-1 production (by mechanism of cross-linking); blocking B and T cells activity. Their removal, in fact, by immunosorbent columns, may stimulate anti idiotypic antibodies production to neutralize and eliminate PDIg from circulation; to inhibit syntesis and increase clearance of CIC that sensitize platelets. We report our experience about six patients with ITP (F/M 5:1, median age 40 years) four splenectomized and refractory to usual immunosoppressive therapy. We used PROSORBA column (IMRE' Corp. Seattle, Wa) containing 200 mg of Staphylococcal protein A that covalently binds 300 mL of silica matrix; so it eliminates circulanting immunocomplexes (CIC) and immunoglobulin G (subset 1,2,4) from plasma according to protein A affinity to their Fc fragment. The mean count of platelets before each treatment amounted to 18.5×10^9/L and in four cases hemorragic manifestations were present. The mean number of proceedings for each case was 4.5, with 3975 mL mean plasmatic treated volume. The treatment carried out by ON-LINE procedure (by means of cellular separator) in two cases and by OFF-LINE procedure in four cases. The period of observation varied from 6 to 15 week. Standards of response were: GR (good-response) PLT >100x10^9/l, FR (fair response) Plt 50<>100×10^9/L, NR (non responder) Plt < 50×10^9/L. We obtained 50% of response (1FR+2GR), so we also observed a disappearance of PAIg (platelet-associated Ig, tested in cytofluorimetric analysis), and CIC. In conclusion, the immunosorbent technique with PROSORBA column results easy executable, without serious collateral effects, not expensive, if it is compared to IVIG (intravenous immunoglobulin) and it is effective in about half of patients.

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DISINTEGRINS ARE POTENT PLATELET AGGREGATION INHIBITORS

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Disintegrins are naturally-occuring polypeptides derived from viper venoms that inhibit the binding functions of integrins; they are potent platelet aggregation inhibitors. Disintegrins are highly homologous proteins; they all contain the arginine-glycine-aspartic acid (RGD) recognition sequence which is presents on several adhesive proteins, such as fibrinogen, fibronectin, vitronectin, von Willebrand factor, collagen, laminin, and thrombospondin. Disintegrins can block the adhesive functions of RGD-dependent integrins such as the platelet fibrinogen receptor, αIIbβ3.
Platelet aggregation is a key event in thrombus formation: it is mediated by the interaction of platelet αIIbβ3 with plasma fibrinogen, and leads to the formation of platelet-rich clots; thus, the inhibition of fibrinogen binding to αIIbβ3 seems an axcellent approach for therapeutic intervention in thrombotic diseases.
Disintegrins are up to 1000 times more potent than linear RGD-containing peptides in inhibiting platelet aggregation: the conformation of the RGD amino acid sequence within the disintegrins likely accounts for their potency.
Disintegrin administration *in vivo* in animals inhibits fibrinogen binding to platelets and platelet aggregation, and increases bleeding time. Infusion of disintegrins in animal models of coronary thrombosis accelerates the rate and extent of thrombolysis and prevents acute thrombotic reocclusion; moreover, disintegrins prevent platelet adhesion and fragmentation in extracorporeal circuits. The antithrombotic effects of disintegrins are dose-dependent and reversible, allowing careful clinical titration of dosage and control of potential bleeding.
Disintegrins are members of a class of proteins found in snake venoms that inhibit platelet aggregation by antagonism of fibrinogen binding to αIIbβ3; when administered in conjunction with thrombolytic agents they may also positively influence thrombolysis. Furthermore, disintegrins serve as model for the design of new potent and selective αIIbβ3 antagonist molecules that may be used as antiplatelet agents.

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ANTIPLATELET ANTIBODIES IN ACUTE AND CHRONIC IMMUNE THROMBOCYTOPENIC PURPURA OF CHILDHOOD

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Immune thrombocytopenic purpura (ITP) is a relatively common hematological problem, including at least two distinct clinical entities. Acute ITP (a-ITP) is predominantly a disease of childhood; spontaneous remission usually occurs within 6 months from the onset. Chronic ITP (c-ITP) is primarily a disease of adults; patients rarely undergo complete remission and almost always require therapy. In the present study, we have evaluated serum samples from pediatric patients with a-ITP or c-ITP for the presence of platelet-reactive antibodies by immunofluorescence (PSIFT) and a solid-phase test (SPRCA). PSIFT platelets were pretreated with paraformaldehyde to reduce nonspecific binding of IgG-Fc to platelet Fc receptor; thus, they are mainly available to interact with the $F(ab')_2$ domain of IgG. Conversely, SPRCA platelets were still available to both Fc- and $F(ab')_2$-mediated interactions. We could define how serum antibodies bind to normal platelet membrane as follows:
PSIFT– SPRCA–: absence of platelet-reactive antibodies.
PSIFT+ SPRCA+: autoantibodies bound via $F(ab')_2$ to platelet membrane; however, we cannot rule out a contemporary presence of Fc-mediated IgG binding.
PSIFT– SPRCA+: presence of IgG bound via Fc to platelet membrane (immune complexes or IgG aggregates).
PSIFT+ SPRCA–: we hypothesize the presence of antiplatelet autoantibodies.
On the basis of our results, we can recognize two different ways by which patient serum antibodies and normal donor platelets may interact: one occurs by a $F(ab')_2$-mediated binding, whereas the second takes place by an Fc-mediated binding. In a-ITP at onset we found prevalence of Fc-mediated binding as compared with $F(ab')_2$-mediated ones (30.8% vs 19.2%); on the other hand in c-ITP at onset $F(ab')_2$-mediated binding were detected more frequently than Fc-mediated binding (41.7% vs 16.7%). Our data suggest that at least two different pathogenetic mechanisms may be responsible for thrombocytopenia in pediatric patients affected with a-ITP or c-ITP.

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THROMBOMODULIN LEVELS IN THROMBOTIC THROMBOCYTOPENIC PURPURA PATIENTS

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Thrombotic thrombocytopenic purpura (TTP) is a rare disease whose characteristics signs and symptoms, microangiopathic anemia, consumption thrombocytopenia, neurologic impairment, fever and renal failure are caused by a widespread microthrombosis. Etiology is unknown even if endothelial damage has been suggested by many Authors.
Thrombomodulin (TM) is a glycoprotein of the endothelial cell surface and it is involved in the protein C anticoagulant pathway.
Recently it has been suggested that increased levels of plasma TM reflect injury of endothelial cells. On this basis this molecule is considered a marker of vascular disorders.
In this study we tested plasma TM levels in six TTP patients either with active disease or in complete remission.
Preliminary data show that TM is always in the normal range. This finding suggest that the endothelial involvement has not a predominant role in the onset of the disease in these patients. However, further studies are needed to confirm this result.

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INCIDENCE AND CLINICAL RELEVANCE OF NON-INHIBITORY ANTIBODIES AGAINST FACTOR VIII IN HEMOPHILIA PATIENTS

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The occurence of antibodies (Abs) capable of inhibiting factor VIII (FVIII) coagulant activity is a severe complication in hemophilia A accounting for 5-20% of transfused patients. Since the assay employed in the screening and assessment of anti-FVIII Abs (Bethesda assay) is conceived on a functional basis, it is not known whether and to what extent post-transfusion antibodies against non coagulant epitopes may arise. In fact, FVIII deficiency should imply that hemophiliacs are not immunologically tolerant to the whole or to the mutated portion of the molecule. Thus, we set up a system capable in theory to detect all the FVIII-induced antibodies by the use of an enzyme-linked immunoassorbent assay (ELISA). The method consisted in coating FVIII obtained from Hemofil M onto polyestirene microtiter plates. Several dilutions of serum samples were added to the wells and, after incubation and subsequent washing, a peroxidase-conjugated rabbit anti-human IgG was used. Serum samples from 45 patients affected by Hemophilia A of different gravity were analysed for the presence of FVIII Abs and none of them had showed any inhibitor titer in Bethesda assay except for one. We detected the presence of non-inhibitory Ab in about 20% of the patients tested; the titer was quite variable and could be related to the time of the last transfusion but not to the total number of them.
The clinical relevance of these antibodies is not known but the formation of immune complexes (Ab-FVIII) might reduce the half-life of transfused FVIII concentrate in so far as it is cleared by reticulo-endothelial system. We challenged this hypothesis in three hemophilia A patients (two with Abs) by administering 30 U/Kg of body weight and determining plasma FVIII at different time intervals in order to measure the recovery of the molecule. While half-life did not show any change in the patient without Abs, FVIII clearance was found markedly reduced in one of the patients displaying high titer Abs.
Our results suggest that a larger number of hemophilia A patients are immunologically tolerant to FVIII in respect to what observed from the incidence of inhibitors. Considering that FVIII molecule is likely to be processed for presentation to T lymphocytes, the number of responders feasible to be detected could be higher if Abs against internal epitopes of FVIII were explored. In this regard, studies are in progress in our laboratory to develope an ELISA for the detection of antibodies against peptides obtained from cyanogen bromide-digested FVIII.

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RAPID MULTIMERIC ANALYSIS OF VON WILLEBRAND FACTOR BY HIGH VOLTAGE VERTICAL ELECTROPHORESIS AND IMMUNOENZYMATIC DETECTION

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Plasma von Willebrand factor (vWf) consists of a series of multimers of different molecular weight; multimeric analysis of plasma vWf is an important diagnostic tool in characterizing von Willebrand disease.
The original technique utilized an SDS-agarose gel electrophoresis to separate vWf multimers, followed by reaction with radiolabeled antibody and autoradiography; subsequently, several methods were developed to avoid the need for radiochemicals and to improve multimer resolution and visualization, including luminography or immunoenzymatic detection.
Here we present a rapid, sensitive technique to analyze vWf multimeric composition. High gelling temperature agarose was used to prepare vertical discontinuous slab gels, using a flexible plastic film as gel support medium. Different resolution of vWf multimeric structure (low, intermediate, high) was obtained varying agarose concentration. Electrophoresis of diluted plasma samples was carried out at 25 mA constant current for 40 min to move samples out of the wells, then it was continued at constant 150 volts for 5 h. Electrophoresis temperature was cooled at 12°C. After electrophoresis the agarose gel was stripped off support medium film using a thin wire, prior to electrophoretic transfer onto nitrocellulose at 450 mA constant current overnight.
The nitrocellulose membrane was blocked in 5% milk, then incubated for 3 h in rabbit anti-human vWf, and subsequently incubated for 2 h in alkaline phosphatase conjugated anti-rabbit IgG. NBT/BCIP was used as chromogenic substrate.
The described procedure appears to be as sensitive as autoradiography and luminography without the disadvantages of the latters; moreover, it requires only 2 days, whereas other techniques are time-consuming (3-5 days).

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EVIDENCE OF CYTOKERATINS PRODUCTION BY THE LEUKEMIC CELL LINE K-562

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The presence of cytokeratins in the supernatant of two continuous leukemic cell lines K562 and CEM has been tested by using two different techniques of radioimmuno-analytic techniques: with polyclonal antibody against polypeptidic tissue antigen (TPA) and with monoclonal antibody againts cytokeratins 18 and 19 (TPS).
The cytokeratin release has been measured in liquid culture medium (in duplicate), during the two different growth phases of the K562 and CEM lines (logarithmic and plateau); the clonogenic assay in semi-solid medium (MEC) has been performed at the same time from the beginning to the 500th hour of liquid culture, every 12 hrs until the 4th day, then every 24 hrs.
While no cytokeratin release has been found in the supernatans of the lymphoid CEM line, a significant level of cytokeratins was always present in the supernatants of the K562 myeloid cell line.
The latter proved to be significantly correlated both with the total cell amount (R=0.94 for TPS; R=0.92 for TPA) and even more so with the dead cell count (R=0.97 for TPA; R=0.93 for TPS).
On the contrary no significant correlation has been found with the total amount of viable cells excluding Trypan Blue, while an inverse correlation was observed with the clonogenic efficiency in the different growth phases.
The incubation of K562 cells with VP16 for 60' at a concentration of 120 mM ($DL_{>90\%}$) induced a more than 15-folds increase in TPS/TPA concentrations, with a complete inhibition of leukemic growth both in the semisolid assay and in liquid culture.
The cytokeratin concentration increase in the supernatant proved to be independent of the accumulation effect (corrected for D cell concentration and D determination interval) only for TPS (R=0.85).
Our experimental model confirms the release of cytokeratins by K562 leukemic line suggesting that K562 cells originate from very immature cells with aberrant phenotype (myeloid, erithroid, megakaryocyte and even epithelial phenotype).
Finally the kinetic of the cytokeratins release suggests that the amount of this product is probabily not correlated with the cell proliferation, but rather with the apoptosis.
The *in vitro* pharmacological treatment confirms that the TPS/TPA release increases in parallel with the cell damage, as shown by Trypan Blue incorporation and by cell growth inhibition in the semi-solid assay.

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CYTOGENETIC AND FISH CHARACTERIZATION OF A NB4 CELL LINE BEFORE AND AFTER GROWTH IN THE SCID MURINE MODEL

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The NB4 cell line has been previously established from a human acute promyelocytic leukemia, M3 (*Lanotte et al., Blood 1991, 77, 1080*). The published karyotype shows an hyperdiploid modal number with structural rearrangements, including unidentified markers; a 12p+ chromosome; two typical translocations 15;17; and derived chromosomes from n.18 and 19.
We recently performed cytogenetic studies to characterize NB4 cells before and after injection into a SCID murine model. Karyotypes were analyzed after G banding with Wright stain. Chromosome painting with a library for chromosome n.15 was also performed to further characterize 15;17 translocations.
Results from cytogenetic studies showed identical karyotypes in NB4 cells before and after injection into the mouse. We found an hyperdiploid modal number with two 15;17, a 12p+ marker, and a der(19), as previously shown. We were also able to identify some markers, such as a 4q- chromosome; a 8q+; a der(11), due to double translocations to both the short and long arm; a 14p+. Furthermore, the previously described der(19) was identified as a translocation between the short arm of chromosome 19, and the long arm of chromosome 10.
Finally one additional marker was a 15p+. Interestingly, chromosome painting showed that at least part of the extramaterial on the short arm of the 15p+ marker was also derived from n.15, q21-qter.
Cell lines originated from malignant cells are a unique tool to study the mechanisms of leukemogenesis. Knowledge of the genetic rearrangements occurring in these cell lines is critical to interpret the biological events.

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GRANULOCYTES' ADHESION MOLECULES IN MYELODYSPLASTIC DISEASES

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Preliminary studies have suggested that granulocytes (PMN) membrane antigens are abnormal in MDS for arrest of maturation and persistence of immature myeloid antigens.
The aim of the present study was to evaluate granulocytes' phenotype in 20 patients suffering from MDS and correlated it with their dysfunction.
We collected eparinized whole blood and we evaluated the CD11/CD18 complex leukocyte adhesion molecules of granulocytes by monoclonal antibodies (MAbs). We observed that the function of PMN in our patients are abnormal. In the same patients we demonstrated defect of chemotaxis, of aggregation and of production of superoxide anion from neutrophils.
Cytofluorimetric analysis showed decreased expression of CD11b/CD18 receptor as detected by OKM1 ($p<0.001$) and of CD18 as detected by MoAb IOT-18 ($p<0.001$). Immunoistochemical analysis by alkaline phosphatase (APAAP) and sequential image analysis study demonstrated the same results and we observed decreased expression of CD11b/CD18 in granulocytes from MDS compared to controls ($p<0.001$).
The complex CD11/CD18 have an important role in the process of development of myeloid cells probably regulating the interaction between cells and endothelium.
PMN of this patients are often immature cells with impaired function and patients have history of recurrent infections.
We thought there is a correlation between decreased expression of adhesion molecules and deficit of granulocytes function. The inability of this cells to interact with microenvironment compartment plays an important role to block maturation and function of PMN predisposing MDS patients to bacterial and fungal infections.

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MEASURING SC5b-9 (TERMINAL COMPLEMENT COMPLEX) IS A USEFUL TOOL IN MONITORING NEUTROPENIC PATIENTS AT RISK OF INFECTION

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The terminal complement complex (TCC) is a macromolecolar complex comprising C5b, C6, C7, C8 and C9 units which are linked on the cellular membranes when the terminal pathway of the complement system is activated. A plasmatic protein removes from the membranes the TCC which become detectable in the plasma in its soluble form (SC5b-9). The potential role of TCC in human disease characterized by acute and chronic inflammation or tissue necrosis, has been investigated. The observation of a significant increase of TCC plasmatic levels in baboons treated with large amounts of Escherichia Coli or endotoxin suggested possible involvement of TCC in the pathogenesis of septic shock. These values correlated with the clinical findings and the IL-1 or TNF-α plasma levels. Furthermore, sporadic evidence of high TCC plasma levels has been reported in patients affected by ARDS or septic shock. In our experience we have tested TCC plasma levels in 19 patients (mean age 41±19) affected by hematologic malignances (12 ANLL, 5 ALL, 1 NHL and 2 HL), with a follow-up of 36±21 days. We collected plasma-EDTA and serum samples from patients every two days during the period of hospitalization when they received chemotherapy and underwent severe neutropenia (PMN < 500 μL) for 18.6±9.1 days. All patients were submitted weekly to surveillance cultures (nasal, rectal and oral swab, urinocolture) and to extensive serologic monitoring; in the case of fever up to 38°C blood cultures were collected. Plasma samples, centrifuged and stored at –30°C within 2 hours from the collection, were tested for SC5b-9 by using a double-sandwich ELISA method and the monoclonal antibody MCaE11 (Diatec). Moreover plasma and serum samples were used to measure the following parameters: C3 and C4 levels, circulating immune complex, IL-1, TNF-α, IL-6. Twenty plasma samples from normal donors were tested as controls. Sixteen out of the 19 patients presented at least one febrile episode over 38°C. Two patients showed a septic shock and two others presented a massive tumor lysis. One patient suffered from an ulceration of the right pharingeal tonsil. In another patient a malacic lesion in the brain complicated the clinical course, while in another case cutaneous necrotic lesions, diffusion of a lung aspergillosis, was observed. The remaining 2 patients did not show any infective or necrotic complications and TCC plasma levels remained in the normal range. A sudden increase in the plasmatic TCC concentrations, up to 7-8 fold, was observed during the developing of the necrotic phenomena and 4.1±2.2 days before the rise of the cutaneous temperature. The TCC levels ran down before the clinical picture became normal. TCC plasma levels were not influenced by the administration of anti-tumoral agents, antibiotics, intravenous immunoglobulins or growth factors. Plasmatic TCC concentration appears a sensitive test to *predict* a possible septic event in neutropenic patients. We postulate that TCC can be involved in the early phases of the inflammatory response to the infections. On the clinical ground monitoring TCC plasma levels could be a precocious and useful marker of infection in susceptible patients.

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PINEAL GLAND AND MALIGNANCY: DAYTIME CIRCULATING LEVELS IN MULTIPLE MYELOMA

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Melatonin (MLT), the main hormone produced by the pineal gland, has been seen to play a role in antineoplastic activity either by exerting a direct inhibitory effect on cancer cell growth, or by stimulating the immune system. Moreover, MLT blood levels have been shown to be increased in cancer patients. Plasma MLT concentrations (pg/mL) were determined in 20 patients with multiple myeloma (MM) and in 20 age matched healthy subjects (61.1±7.4 vs. 58.1±15.7; $p = 0.4$). Venous blood samples were drawn between 8 and 9 a.m. from February to April 1993 and MLT was assayed using a commercially available radioimmunoassay (RIA) procedure (kit N.I.D.S., S. Juan Capistrano, CA, USA). The data were analyzed by Student's t-test and results are reported as mean values±standard deviation (SD). The patients with MM showed significantly higher mean MLT serum levels than healthy subjects (29.1±14.3 vs.14.3±4.9; $p < 0.001$).
MLT secretion is influenced by manyfold factors and it's periodical in seasonal, monthly, ultradian and, prominently, in circadian frequency domain. The most powerfull synchronizer of MLT circadian rhythm is ligth/dark cycle: rhythm acrophase can be found half-way through the sleeping hours.
Our subjects, patients and controls, have been sampled at the same hour from awaking in order to obtain an apparently comparable circadian synchronization. This pineal response could actually be a phenomenon secondary to an altered endocrine-metabolic balance caused by an increased demand of the developing tumor.
As an alternative the enhanced MLT secretion might be considered as a compensatory mechanism because of its antimithotic action or interpreted as an effort to secrete substances capable of regulating neoplastic growth.

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PRIMITIVE PANCREATIC LOCALIZATION OF NON HODGKIN LYMPHOMA: TWO CASES

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We report the cases of two patients who underwent surgical procedures for the suspicion of primitive pancreatic carcinoma and whose hystologic biopsy demonstrated a non Hodgkin lymphoma.
The first patient, a 74-year-old man, was admitted to the surgical unit because of weight loss and abdominal pain. The physical examination showed icteric, the blood test a ESV of 30 mm/h; a total bilirubin 7,30 mg/dL with a direct bilirubin 5,20 mg/dL, an alcaline phosphatase 846 U/L (upper limit 300); GOT 198 U/L (upper limit 34); GPT 273 U/L (upper limit 33); GGT 530 U/L (upper limit 49); amylase 216 U/L (upper limit 195).
Abdominal imaging studies (c.e. ultrasound scan and CT scan) showed dilation of the intrahepatic ducts and of the common bile duct; a solid mass in the head of the pancreas without any involving of abdominal lymph nodes. A biliary diversion was performed together with a biopsy of the pancreatic mass which showed an intermediary malignancy non Hodgkin lymphoma. The research of other lymphoma localizations, research made by gastric endoscopy, thoracic CT scan and bone marrow biopsy, was negative.
The second patient was a 46-year-old woman admitted to the surgical unit for epigastric pain. The blood test showed amylase 1038 U/L and ESV 32 mm/h. The imaging studies showed a solid mass in the head of pancreas without any lesions to the liver, spleen, kidneys and adrenal glands. A diagnosis of pancreatic carcinoma was made and the patient underwent a duodenocephalopancreatectomy. Afterwards the biopsy showed a centrocytic centroblastic lymphoma with a low grade of malignancy. Again the research for other lymphoma localizations was negative.
Thus, it is possible a primitive pancreatic localization of non Hodgkin lymphoma without any other involvement and this should be considered in the differential diagnosis of a new pancreatic mass; with regard to this we strongly advise an intraoperative biopsy of any pancreatic mass avoid diagnosis and treatment mistakes.

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67-GALLIUM PLANAR AND SPECT SCINTIGRAPHY, COMPUTED TOMOGRAPHY (CT) AND MAGNETIC RESONANCE IMAGING (MRI): STAGING AND FOLLOW-UP OF MALIGNANT LYMPHOMAS

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In this study we compared the results of planar and SPECT 67-Ga scintigraphy with CT and MRI in patients affected by lymphomas to evaluate the utility of different imaging techniques in the staging and follow-up. Since May,1993, we evaluated 30 patients (14 males and 16 females) affected by HL (17) and NHL (13); median age was 31.03 years (range 14-46). Overall forty-four 67-Ga scan (planar and SPECT), 44 CT and 35 MRI were performed before treatment (13 patients) and at least one month after the end of therapy (follow-up 6-24 months). Scintigraphic images were obtained 48 hours after i.v. injection of 370 MBq 67-Ga. Planar scintigraphy whole-body views were obtained; thoraco and abdominal SPECT were performed after planar studies. Standard non-contrast and contrast CT scans were carried out in all patients,with contiguous,1-cm-thick sections. MRI was performed in 25 patients who received 2 spin-echo sequences with cardiac gating, one T2-weighted and one T1-weighted sequences, with an 8 mm slice thickness and 2 mm gap. All the three imaging studies were obtained within one week. All the studies performed at the initial staging prior to therapy (13 67-Ga planar and SPECT, 13 CT and 10 MRI) were positive. After treatment out of 31 planar 67-Ga three were positive, 24 negative and four equivocal; out of 31 SPECT 67-Ga ten were positive,18 negative and three equivocal. Four negative and three equivocal planar images became positive at the SPECT study. Out of 31 CT, 12 were positive, 15 negative and four equivocal.
Out of 25 MRI, 13 were positive and 12 negative. In two patients with residual mediastinal mass greater than 2 cm and in one patient with post-therapy diffuse lung disease CT and MRI were positive while 67-Ga planar and SPECT were negative (three false-negative). In three post treatment equivocal 67-Ga SPECT studies we found some abnormal ilar uptake without corresponding CT and MRI abnormalities. This pattern, whose etiology remains unknown, cannot be attributed to lymphoma unless confirmed by other methods. At present, CT and RMI are the most useful imaging techniques for the initial staging of lymphomas, but 67-Ga scintigraphy should be performed before treatment as a baseline study to identify patients with Gallium-avid tumors. Only these patients should be included in the follow-up with 67-Ga scintigraphy. The sensitivity of 67-Ga SPECT is better than planar images: lesions undetected or equivocal with planar scintigraphy could be obvious on SPECT. 67-Ga SPECT and MRI appear to have the same sensitivity in evaluating the presence of viable tumor in residual mass after treatment, whereas CT could be inconclusive. Furthermore, 67-Ga whole-body scintigraphy is useful to identify unknown localizations of disease and to detect relapse in new regions as compared to primary site involvement.

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SPLENOMEGALY AND NON-HODKGIN LYMPHOMA. ULTRASONOGRAPHY EVALUATION AND POSSIBLE APPLICATIONS

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The increase in spleen volume during chronic lymphoproliferative disease is related to the expansion of lymphoid component. Less than one third of patients affected by non Hodgkin's lymphoma (NHL) present with clinically detectable splenomegaly. This study aimed at identifying new ultrasonography (US) parameters capable of precisely estimating spleen volume and weight in healthy subjects and in patients affected by NHL. US was carried out on 11 *postmortem* spleens weighing between 30 and 2230 g.
The organs were examined in a water bath and the maximum splenic area on the coronal plane resulted to be the parameter most correlated with spleen weight (r=0.98). Subsequently, the spleens of 37 voluntary healthy subjects were submitted to US evaluation. In this group, the splenic area measured 34±8 sq.cm., equal to 221 g. The spleens of 10 patients affected by NHL were evaluated by US against these reference standards. Examination were carried out at the onset of the disease, during chemotherapy and at the end of the treatment. In patients with NHL, the mean value of the splenic area was 43±22 sq.cm, differing in a statistically way from the value found in healthy subjects ($p<0.004$). In 5 patients with NHL, US showed larger spleen than the normal average. Splenomegaly was recognizable by manual examination in only one. In all patients with US detected splenomegaly, a tendency to splenic area decrease was observed during chemotherapy. The two cases with normal spleen volume also showed a decrese of the splenic area during treatment.
Conclusion. The US evaluation of the maximum splenic area on the coronal plan is a reliable method to measure weight and volume and can be useful in patients with NHL to identify mild splenomegalies that are not clinically detectable, and to single out volume decreases in the various stages of chemotherapy and follow-up, even in relatively *normal* spleen.

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PEDIATRIC NON HODGKIN-LYMPHOMA: A RETROSPECTIVE ANALYSIS OF UNICENTRIC EXPERIENCE FROM 1961 TO 1992

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Introduction. Pediatric non-Hodgkin lymphomas (P-NHLs) constitute a heterogeneous group of malignant diseases wich account for 7-10% of all childhood cancer and are third in relative frequency after leukemias and brain tumours. With respect to adult-NHLs, P-NHLs differ in frequency, biology, clinical features, histology, prognostic factors and treatment. In the last twenty years (yrs) major improvments has been made in the choice and results of treatment. In order to assess the change of efficacy of several protocol adopted we carry out a retrospective study on our own thirty years experience in the P-NHLs treatment. *Patients.* All cases of P-NHLs, aged 0-15 and diagnosed from 1961 to 1992, were evaluated. Since 11 type of chemotherapy protocols were used during the study period they have been divided in 4 main groups (BFM, PD 79, COPAD-VCAD, and others) for the efficacy analysis. *Results.* During the study period 127 patients (pts.) were diagnosed affected by P-NHL. Demographics data showed a median age of 7 yrs. and a male/female ratio of 3:1. The main aspects was the followings (number of pts. suitable for evaluation in brackets): stage (112) I 19%, II 4%, III 54%, IV 23%; histology (117) small non cleaved 49%, lymphoblastic 27%, large cell 9%, others 15%; primary disease site (118) abdomen 33%, mediastinum 30%, peripheral nodes 19%, pharinx 9%, bone 4%, skin 3%, miscellaneous 2%; immunophenotype (84) B 60%, T 30%, non B non T 10%. *Chemotherapy response:* first complete remission (CR) was obtained in 83% ps. (106/127) with median time of 42 days, and 57% pts.(72/127) completed the assigned protocols. Thirty nine pts. (30.7%) relapsed (31 in therapy and 8 off-therapy) and only 16 pts. obtained a 2nd CR. In this group, 7 pts. achieved the stop-therapy: they were 4 relapsed in-therapy and 3 relapsed off-therapy respectively. *Statistical analysis of prognostic factors.* Event free survival (EFS) was affected in statistical significance manner by stage at diagnosis (EFS=83.9, 80, 57.7, 17.1% respectively for I, II, III, IV), immunophenotype B and non B-non T vs. T (EFS=65, 68.6, 36.9% respectively), type of protocol, BFM and PD 79 vs COP-VCA and OTHER (EFS=73.4, 64, 29.4, 22.4% respectively) whereas sex and age did not. Survival (SUR) by protocol was 4.1, 65.3, 32, 27.2% and disease-free survival (DFS) was 76.5, 67.2, 33.3 and 38,7% respectively for BFM, PD 79, COP-VCA and OTHER protocols. *Discussion.* The primary treatment modality for P-NHLs is chemotherapy whereas radiotheray has only an ancillary role such as emergency treatment (i.e., superior vena cava compression). The improved survival obtained in the last twenty yrs. is related to the design of well standardized and more intensive chemotherapeutic regimens as provided by BFM and PD 79 protocols. This choice has increased the survival from 27-32% to 65-84% of pts. Moreover, high dose of chemotherapy with autologous bone marrow transplantation or peripheral blood staminal cells rescue is considered at present the only way to increase the survival in the pts. with poor response to primary chemothery or relapsed. In this context, the carefully study and determination of prognostic factors are of important value in order to identify the poor-prognosis pts. and avoid to give unnecessary toxicity to good-prognosis pts.

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FACTORS PREDICTING KARYOTYPIC RESPONSE IN PATIENTS WITH CHRONIC MYELOID LEUKEMIA TREATED WITH ALPHA-INTERFERON

Eliana Zuffa for the Italian Cooperative Study Group on Chronic Myeloid Leukemia

α-interferon is currently believed to have the potential of modifying the course of chronic myeloid leukemia. The Italian Cooperative Group enrolled 322 patients in a prospective study between 1986 and 1988: 218 pts randomized to IFN and 104 to conventional chemotherapy.
The rate of karyotypic response (defined as >33% of Ph negative metaphases) was 30% in the interferon group and 5% in chemotherapy group ($p<0.001$). Among the karyotypic responses, 19 were *complete* (100% Ph neg), 24 *major* (66-99% Ph neg) and 24 *minor* (33-65% Ph neg). Survival seems to be related with karyotypic response (KR): patients with *complete, major* and *minor* KR have a survival rate of 82% after 72 months, vs 36% in minimal or no KR and 30% in CHT group. 47/67 (70%) patients with a *good* KR had same degree of response at 1 year, 12 (18%) at 2 years and 8 (12%) after 2 years of treatment.
Since interferon treatment is very expensive, may have important side effects, and must be prolonged for several years, it is important to identify features that might predict karyotypic response and survival. We analyzed by multivariate logistic regression many clinical parameters at diagnosis. Karyotypic response occurred more often in patients with a normal platelets count ($p=0.02$) and with a very few or not detectable blast cells in peripheral blood ($p=0.009$). Spleen size and risk group (Sokal relative risk) were not significantly related to response.
Hematological response is a feature with prognostic value: karyotypic response occurred in 50% of patients with hematological response after 8 months of treatment, versus 15% of patients with no hematological response ($p=0.002$).

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α-INTERFERON (IFN) THERAPY IN ESSENTIAL MIXED CRYOGLOBULINEMIA

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Essential mixed cryoglobulinemia (EMC) is a chronic vasculitis due to cryoprecipitable immunocomplexes consisting of policlonal IgG and monoclonal IgM rheumatoid factors. The monoclonal component is, at the same time, the expression of an autoimmune phenomenon and of a low-grade clonal lymphoid proliferation. In recent years a role for hepatitis C virus(HCV) has been suggested in the pathogenesis of the disease, and antiviral α-INF therapy is considered beneficial in several reports, on clinical ground. We describe 5 patients, (4 women and 1 man), with EMC resistent to previous conventional therapy (steroids, cyclophosphamide), treated with α-INF, to outline difficulties aroused in their management. A woman was 36 years old, the others were over 65. All of them presented fever, arthralgia, leukocitoclastic purpura. Three had HCV antibodies. HCV RNA was detected in 2 out of 3 sera tested with polymerase chain reaction essay for 5'UTR region. α-INF was permanently discontinued in 3 instances within one month, because of an acute adult respiratory distress syndrome in 1 case, spiky fever and relapsing arthritis despite maximum paracetamol coverage in another and ascites and cachexia in the third suffering of chronic liver disease. Two patients received α-INF thrice weekly for 6 months (6 MU and 3 MU respectively, in the second case with low dose of steroids).
On one hand symptoms and signs of disease didn't improve substantially. On the other hand we noted an increase of serum IgM component, from medium values of 234 mg/dL to 556, and of rheumatoid factors, from 383 UI/mL to 1050 at the end of treatment. No other autoantibody was detected. α-INF has antiviral and antiproliferative proprieties that support therapeutic indications. α-INF interaction with the immune system is less well known, but it definitely exist; it may induce autoantibodies production or make worse autoimmune hepatitis.
Our cryoglobulinemic patients demonstrate low tollerance to the INF therapy, 2 of them developed highly risky conditions and the 2 treated for 6 months didn't show subjective improvement while the serum autoimmune phenomena increased.
Our experience do not conferm positive results obtained by other authors. We suggest that α-INF, in an already altered immunoregulatory system, may enhance immune disorder and autoantibodies production.

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LONG-TERM RESULTS IN THE TREATMENT OF HAIRY CELL LEUKEMIA WITH α-INTERFERON

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Hairy cell leukemia (HCL) is a chronic lymphoproliferative disease of B lymphocytes blocked in an advanced stage of differentiation, morphologically characterized by prominent cytoplasmic protrusions. The immunophenotypic feature is the double positivity to CD19 and CD25 and the presence of interleukin-2 receptor (IL2R) which is released in high amount in the serum as a soluble form (soluble IL2R). Bone marrow infiltration is accompanied by variable amount of fibrosis.
α-interferon (α-IFN) have been the best therapeutic approach in the last decade. We have assessed our results on the verge of the introduction of new effective agents in the treatment of HCL. Patient were to receive recombinant α-IFN (either 2a or 2b) 3×10^6 UI subcutaneously by a three times a week schedule for 52 weeks; a second course of the same treatment was initiated at the time of progression.
Nineteen patients (17 male and 2 female, mean age 60.6 years) were then treated at our institution from 1986 to 1993. These patients have received α-IFN for a mean period of 23.3 (in. 6, max. 59) months. After 12 months of therapy the levels of Hb have had an increase of 25%, the WBC count of 42%, the platelet count of 45%. The bone marrow infiltration was reduced by 47% and the splenomegaly present at the diagnosis in 14 patients disappeared in 10 out of 14 cases. The mean follow-up is 38.8 (min. 12, max. 87) months.
None of the patients have obtained a complete remission, 12 patients (63%) had a partial remission (the bone marrow infiltration was reduced of 15% in the best three cases); 5 patients had a minimal response (stable disease) and only 2 were resistant to the therapy. Such results have not changed in the three following years with a mean period free of treatment of 12.6 months. The mean survival at 5 years is 94.7% (one patient died of lung cancer). Side effects have been low: severe, reversible neutropenia in two cases and a diffuse macular erythema promptly regressed by steroids in two other patients.
α-IFN response showed to be effective for long time in most of HCL patients but a higher percentage of complete remission could be reached only by increasing the dosage.
In the light of our results, α-IFN will keep its role in the treatment of HCL if compared to the overall toxicity of the more recent purine-analogs.

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FLUDARABINE THERAPY IN DIFFERENT CHRONIC LYMPHOPROLIFERATIVE DISEASES: RESULTS IN 28 PATIENTS

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The aim of the study was to evaluate the effect of fludarabine (Fluda) on the outcome of 28 patients affected by chronic lymphoproliferative diseases (CLDs). The median age was 54 (range 30 to 70 years); 19 patients were male. Diseases, according to the modified Kiel classification, were so distributed: 17 B-CLL, 3 B-PLL, 5 macroglobulinemic lymphomas, 1 pleomorphic immunocitoma, 2 cb/cc follicular NHL. Fifteen patients were untreated, 13 patients were previously treated with regimens including alkylating agents, 8 being resistant. Fluda 25 mg/m^2/day for 5 days to be repeated every 28 days, was administered every month for a median of 4 cycles (range 1 to 6 cycles). Dose reduction to 18 mg/m^2 was supposed in presence of serum creatinina > 15 mg/L and thrombocytopenia (grade III WHO). Twenty seven patients were evaluable for response. Out of the 15 previously untreated patients, 12 (80%) had a response to Fluda: 5 obtained a complete remission (CR) and 7 a partial remission (PR); only 4 (30%) out of 13 previously treated patients achieved a PR. As expected, the better results were observed in the set of untreated patients (12 cases) with CLL (2 CR and 6 PR) and low grade NHL (3 CR and 1 PR).
Neutropenia (grade III WHO) was the most frequent hematological toxicity (15 cases, 55%). Major infective complications were observed in 7 (25%) cases, being fatal in 2. Extra-hematological toxicity with a reversible grade IV WHO increment of SGOT - SGPT developed in one patient.
Our data confirms the efficacy of Fluda in the management of CLDs, especially in CLL. Neutropenia induced by Fluda certainly contributes to the high risk of morbidity and mortality observed in these patients who are older and with a severe immunodeficency.

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COMBINATION OF FLUDARABINE, MITOXANTRONE, AND PREDNISONE IN RECURRENT LOW-GRADE NON-HODGKIN'S LYMPHOMA

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Recently, several new drugs have been identified that appear to be particularly effective against low-grade non-Hodgkin's lymphoma (LG-NHL). Fludarabine (FLU) phosphate is one of these promising purine analogs. As a single agent, FLU has produced responses, mostly partial, in 50% to 60% of patients with reccurent or refractory LG-NHL.
Mitoxantrone, an anthracenedione, presents a promising activity in recurrent lymphomas and particularly in LG-NHL with response rate in 30% to 60% of patients.
On the basis of these promising activity of FLU and mitoxantrone as single agents and of synergistic activity of FLU combined with mitoxantrone in inducing apoptosis, we conducted a study with these two agents an prednisone in combination (FMP). Between November 1993 and May 1994, 12 recurrent LG-NHL, according the Update Kiel classification, received FMP regimen using these doses: FLU 25 mg/m^2/day, days 1-3, mitoxantrone 10 mg/m^2 day 1, and prednisone 40 mg/m^2/day, days 1-5, every 4 weeks for six total courses. There have been 2 (17%) complete remissions and 8 (67%) partial remissions, and the disease progressed in the remaining 2 patients during treatment.
The overall response rate (CR+PR) was 84%. With regard to hematologic toxicity, the neutropenia and thrombocytopenia were responsible for hematologic recovery leading to early discontinuation of courses in 2 patients. Of 3 infectious episodes, all were minor, including two upper respiratory infections and one febrile episode that was successfully managed with oral antibiotics.
The high overall response rate is encouraging to continue this study evaluating the durability of responses and, as second step, to try the role of this regimen in the first-line therapy of LG-NHL patients.

FLUDARABINE AND PREDNISONE VERSUS FLUDARABINE, PREDNISONE AND INTERFERON FOR THE TREATMENT OF CHRONIC LYMPHOCYTIC LEUKEMIA: PRELIMINARY RESULTS OF A MULTICENTRIC PROSPECTIVE RANDOMIZED TRIAL

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Between March 1993 and May 1994, 50 previously untreated B-CLL pts observed at Hematology Institutes of Rome (25 pts), Bologna (11 pts) and Udine (14 pts) were randomized to receive 6 monthly courses of therapy, with either fludarabine (Fluda, Schering SpA: 25 mg/m^2/ iv/ day, days 1-5) and prednisone (PDN: 20 mg/m2/day, days 1; 3; 5; 7; 14 and 40 mg/m^2; days 9-13) or the same therapy schedule associated to eight doses of human lymphoblastoid IFN (Wellferon, Wellcome Foundation Limited, 2 MU/sc/day; every second day from 1° to 15° day).
The median age was 58 yrs (range: 35-65 yrs); 7 pts were Rai stage III - IV and 43 pts Rai stage II with progressive disease. Diffuse marrow involvement was present in 25 pts. The median interval from CLL diagnosis to start of therapy was 4 months (range: 1 - 97 months).
Thirty one of the 50 enrolled pts have been now assessed for response: 18 pts treated with FLUDA + PDN (arm A) and 13 pts treated with FLUDA + PDN + IFN (arm B).
At this time, using NCI criteria for response, the overall response (PR + CR) rate is 81%.
Therapy was administered on an outpatient basis. The major toxicity consisted primarily of myelotoxicity and infections. No neurological toxicity and early deaths were observed.
Our preliminary results suggest that FLUDA + PDN IFN is an effective regimen with acceptable toxicity for young patients with B-CLL. Further follow-up is needed to assess whether the addition of IFN to FLUDA + PDN may improve the quality and duration of response in B-CLL.

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