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Immunology and Leukemias

Groupe d'Etude Immunologique
des Leucémies, Dijon, France
January 31-February 2, 2002

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3. The Royal Marsden Hospital Bone-Marrow Transplantation Team. Failure of syngeneic bone-marrow graft without preconditioning in post-hepatitis marrow aplasia. *Lancet* 1977; 2:242-4.
4. Red cell aplasia (Editorial). *Lancet* 1982; 1:546-7.
5. Karlsson S, Humphries RK, Gluzman Y, Nienhuis AW. Transfer of genes into hemopoietic cells using recombinant DNA viruses [abstract]. *Blood* 1984; 64(Suppl 1):58a.

Books and other monographs [personal authors,^{6,7} chapter in a book,⁸ published proceeding paper,⁹ abstract book,¹⁰ monograph in a series,¹¹ agency publication¹²]:

6. Ferrata A, Storti E. *Le malattie del sangue*. 2nd ed. Milano: Vallardi, 1958.
7. Hillman RS, Finch CA. *Red cell manual*. 5th ed. Philadelphia: FA Davis, 1985.
8. Bottomley SS. Sideroblastic anaemia. In: Jacobs A, Worwood M, eds. *Iron in biochemistry and medicine*, II. London: Academic Press, 1980:363-92.
9. DuPont B. Bone marrow transplantation in severe combined immunodeficiency with an unrelated MLC compatible donor. In: White HJ, Smith R, eds. *Proceedings of the third annual meeting of the International Society for Experimental Hematology*. Houston: International Society for Experimental Hematology, 1974:44-6.
10. Bieber MM, Kaplan HS. T-cell inhibitor in the sera of untreated patients with Hodgkin's disease (Abstract). Paper presented at the International Conference on Malignant Lymphoma Current Status and Prospects, Lugano, 1981:15.
11. Worwood M. Serum ferritin. In: Cook JD, ed. *Iron*. New York: Churchill Livingstone, 1980:59-89. (Chanarin I, Beutler E, Brown EB, Jacobs A, eds. *Methods in hematology*; vol 1).
12. Ranofsky AL. *Surgical operation in short-stay hospitals: United States-1975*. Hyattsville, Maryland: National Center for Health Statistics, 1978; DHEW publication no. (PHS) 78-1785, (Vital and health statistics; series 13; no. 34).

Forthcoming¹³ or electronic material¹⁴:

13. Leshner AI. Molecular mechanisms of cocaine addiction. *N Engl J Med*. In press 1996.
14. Morse SS. Factors in the emergence of infectious diseases. *Emerg Infect Dis* [serial online] 1995 Jan-Mar [cited 1996 Jun 5];1(1):[24 screens]. Available from URL: <http://www.cdc.gov/ncidod/EID/eid.htm>

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table of contents

2002; vol. 87;
supplement to no. 1
january 2002

(indexed by Current
Contents/Life Sciences and in
Faxon Finder and Faxon
XPRESS, also available on
diskette with abstracts)



**Session A
Diagnosis and Techniques**

Tricks and wonders of flow cytometry
D'Hautcourt JL 1

MO acute leukemia
Béné MC, van't Veer M 1

Improvement of the chronic lymphocytic leukemia scoring system using a semi-quantification of fluorescent signals
Brunet C, Cuquemelle C, Potie C, Sabatier F, Camoin L, Conciatori M, Harle JR, Pagniez M, Sampol J, Dignat-George F 1

Routine use of immunophenotype by flow cytometry in tissues with suspected hematological malignancies: role in the diagnosis and detection of double pathology
Villamor N, Martinez A, Aymerich M, Castillo M, Colomer D, Bellosillo B, Campo E 2

CellQuant calibrator: a new flexible tool for routine quantitative immunophenotyping
Teuma X, Brunet C, Lavabre-Bertrand T, Soler F, Dignat-George F, Poncelet P 2

Telomere length of blast cells in acute myeloblastic leukemia determined by flow cytometry
Maynadié M, Lizard G, Girodon F, Mannone L, Carli PM 2

Four intracellular enzymes detected by flow cytometry in the normal bone marrow cell populations separated by the CD45 expression
Maynadié M, Yseabert L, Girodon F, Tatou E, Carli PM, Chatelain B, Béné MC, Faure G, for the GEIL 3

Expression of antigen CD34 and CD71 on the hematopoietic stem cells. Therapeutic interest in tropical urban areas. Abidjan, Côte d'Ivoire
Sawadogo D, Oulai U, Salou M, Sangaré M, Kouassi D 3

**Session B
Therapeutics**

Recombinant antibodies: tailor-made molecular precision tools
Dübel S 3

Generation of potent TH1 responses from patients with lymphoid malignancies after differentiation of β lymphocytes into dendritic-like cells
Mohty M, Isnardon D, Charbonnier A, Lafage-Pochitaloff M, Merlin M, Sainty D, Olive D, Gaugler B 4

Altered HLA expression in non-Hodgkin's lymphoma is frequently associated with aggressive subtype, relapse or transformation
Drénou B, Le Friec G, Bernard M, Pangault C, Grosset JM, Lamy T, Fauchet R, Amiot L 4

Different significance of minimal residual disease after autologous and allogeneic stem cell transplantation for chronic lymphocytic leukemia: prognostic and therapeutic implications
Villamor N, Esteve J, Colomer D, Aymerich M, Rovira M, López-Guillermo A, Carreras E, Campo E, Montserrat E 5



table of contents

2002; vol. 87;
 supplement to no. 1
 January 2002

(indexed by Current
 Contents/Life Sciences and in
 Faxon Finder and Faxon
 XPRESS, also available on
 diskette with abstracts)

Session C
Acute leukemia and apoptosis

Functional features and treatment outcome of T-lineage ALL
Ludwig WD, Schrappe M, Karawajew L5

Involvement of caspases in terminal cell differentiation
Solary E6

Cryptic t(5;14)(q35;q32) translocation in children with t-ALL: immunologic features
Leymarie V, Hélias C, Falkenrodt A, Entz-Werlé N, Lutz P, Mauvieux L, Lessard M6

Expression of BCL-2 family proteins and caspase activities in myelodysplastic syndromes
Boudard D, Viallet A, Chautard S, Piselli S, Mounier C, Vacher JF, Sordet O, Guyotat D, Campos L6

Evaluation on U937 cells of the proapoptotic potency of different oxysterols and of their ability to induce inflammatory processes
Lemaire-Ewing S, Corcos L, Monier S, Samadi M, Guyot M, Kahn E, Néel D, Maynadié M, Solary E, Gambert P, Lizard G7

Immunologic phenotype of childhood acute lymphoblastic leukemia: a study of 49 cases
Laatiri MA, Fekih S, Chehata S, Jenhani F, Ennabli S7

A biclonal acute leukemia (BAL) with a t(4;11) (q2;q23) translocation
Garnache F, Liénard A, Poulet J, Bulabois B, Collonge MA, Plouvier E, Saas P, Darodes de Tailly P7

Expression of molecules of the TETRASPAN family on blast cells from acute leukemia patients
Faure G, Boucheix C, Rubinstein E, Kolopp Sarda MN, Kennel de March A, Béné MC8

In situ identification of oxidative DNA damages in U937 cells treated by various pro-apoptotic compounds using immunofluorescent detection of 8-oxoguanine by flow cytometry
Monier S, Nacol-Lizard S, Chaouchi N, Gambert P, Lizard G8

Session D
Acute myeloid leukemia

Immunophenotypic clustering of myelodysplastic syndromes
Feuillard J, Maynadié M, Picard F, Chatelain B, Dromelet A, Husson B, Campos L, Rosenwadj FM, Lepelley P, Jouault H on behalf of Groupe d'Etude Immunologique des Leucémies (GEIL)9

Immunophenotypic analysis of minimal residual disease in hematologic malignancies: from techniques to clinical utility
Orfao A, Vidriales B, Ciudad J, López-Berges MC, San Miguel JF9

A consensus immunophenotypic panel for leukemia and other hematopoietic proliferations
Davis BH 10

Immunologic classification of acute myeloblastic leukemias
Casasnovas RO, on behalf the GEIL 10

Circulating blood dendritic cells from myeloid leukemia patients display quantitative and cytogenetic abnormalities as well as functional impairment
Mohty M, Jarrossay D, Lafage-Pochitaloff M, Zandotti C, Brière F, de Lamballeri XN, Isnardon D, Sainty D, Olive D, Gaugler B 11

Characterization of PML antigen delocalization in acute promyelocytic leukemia by immunocytochemistry
Goffinet C, Cornet Y, Chatelain B, Bernier M, Ketelslegers O, Hennaux V, Chatelain C 12

Quantitative expression of the three epitopes of CD34 in 300 cases of acute myeloid leukemia
Maynadié M, Gerland L, Aho S, Girodon F, Bernier M, Brunet C, Campos L, Daliphard S, Deneys V, Falkenrodt A, Jacob MC, Kühlein E, Le Calvez G, Moskvotchenko P, Philip P, Carli PM, Faure GC, Béné MC and the GEIL 12



table of contents

2002; vol. 87;
 supplement to no. 1
 January 2002

(indexed by Current
 Contents/Life Sciences and in
 Faxon Finder and Faxon
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Clinical role of acute nonlymphoblastic leukemia blast cells immunophenotype
Volkova M, Tupitsyn N, Markina I 12

CD56+ expression of acute myeloid leukemia may predict fatal outcome in children with Down's syndrome
Sánchez-Ramón S, Rodríguez-Sáinz MC, Cantalejo MA, Cela E, Galarón P, Díez JL, Pérez I, Fernández-Cruz E, Gil J 13

Characterization of an acute leukemia derived from dendritic lymphoid cells with a CD33 expression
Garnache F, Chaperot L, Poulet J, Bulabois B, Darodes de Tailly P, Deconinck E, Saas P..... 13

Immunophenotype of myeloblastic leukemias in the GOELAMS protocols. A prospective study on 450 cases
Béné MC, Faure GC, the GEIL and GOELAMS French collaborative groups..... 14

Morphologic and immunologic characteristics of biphenotypic acute leukemia: a multicentric study of 57 cases
Garand R, Imbert M, Jouault H, Garnache F, Salaun V, Mallet M, Maynadié M, Casasnovas O, Lepelley P, Preudhomme C, Trimoreau F, Arnoulet C, Sainty D, Latger-Cannard V, Buisine J, Pédrón B, Fenneteau O, Daliphar S, Sudaka I, Philip P, on behalf of the GFHC..... 14

Interest of immunophenotyping in acute myeloid leukemia
Dina J, Salaün V, Reman O, Troussard X, Cheze S, Macro M, Leporrier M 14

**Session E
 Lymphoproliferative disorders**

Chronic lymphoproliferative disorders
Matutes E 15

B-cell chronic lymphoproliferative disorders: the GEIL experience
Deney V..... 16

Value of adhesion molecule and BCL-2 expression for the distinction between Burkitt's and non-Burkitt's (or Burkitt-like) lymphomas
Basaggio L, Pages J, Callet-Bauchu E, Morel D, Berger F, Felman P 16

Relation of CD38 expression with diagnosis of de novo chronic lymphocytic leukemia
Garnache F, Billoit M, Poulet J, Bulabois B, Saas P, Brion A, Darodes de Tailly P 17

Simultaneous occurrence of mycosis fungoides/Sézary syndrome and chronic lymphocytic leukemia
Clody P, Parisi E, Servant M, Mazurier I, Grange F, Moskovtchenko P..... 17

Contribution of flow cytometry in the diagnosis of Hodgkin's lymphoma
Goffinet C, Cornet Y, Dromelet A, Chatelain B 18

Clinical and biologic characteristics of two cases of B-cell chronic lymphocytic leukemia with CD8 expression
Dumitrescu AM, Colita DN, Moicean AD, Georgescu O, Niculescu R, Nitu G..... 18

Usefulness of flow cytometry analysis in addition to the morphologic analysis for the B-cell non Hodgkin's lymphoma
Renoir C, Czerkiewicz I, Yacoub M, Dhello G, Breitenbach L, Dioucoure D, Bissieres P, Feuillard J, Martin A, Raphael M 18

Antigen presentation pathway via HLA class II molecules is modified in certain non-Hodgkin's B lymphomas
Magniez N, Garban F, Plumas J, Pasquier MA, Sotto JJ, Roucard C 19

Benign monoclonal T lymphocytes
Escoda L, Ortin X, Cabezudo E, Colomer D, Villamor N, Llorente A, Boixadera J, Bosque G, Ugarriza A 19

B cells of lymphoproliferative disorders express a receptor for the mitogenic heparin affinity regulatory peptide
Brignole-Baudouin F, Achour A, Barritault D, Courty J 19

index of authors.....i

**SESSION A
DIAGNOSIS AND TECHNIQUES****TRICKS AND WONDERS OF FLOW CYTOMETRY**

D'Hautcourt JL
C.H.R. Mons, Warquignies, Belgium

During the last two decades flow cytometry has become a powerful technique for obtaining multiparametric data on a variety of particles or cells. This technology has been transferred from the research community into the clinical arena, where it has become invaluable as a resource for cell identification, population discrimination, cell counting and/or fluorescence quantification. However, despite the rapid evolution of cytometers that can make sensitive and objective measurements of ever more parameters on a single cell, translating descriptions of complex phenotypes into meaningful clinical information has proven no small challenge. Unfortunately, the capability of multiparameter flow cytometry to measure simultaneously four, six and in the near future up to nine fluorescence parameters on stained cells and thus to gain information about their phenotype remains largely underused. There are a number of reasons for this, but the small number of available analysis software currently marketed and the difficulties in visualizing multidimensional space are thought to be the major reasons. Perhaps one other reason is the unconscious wish to maintain some degree of similarity with older methods, such as the microscopic technique, in order to keep references. This presentation will comment on some new approaches in multiparameter flow cytometric analysis and some useful applications of underused parameters.

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Béné MC, van't Veer M
EGIL, European Group for Immunophenotyping of Leukemia.

Acute myeloid leukemia (AML) M0 is a rare disease, comprising about 5% of AML in adults, which has also been reported to occur in infancy and childhood. We report on the hematologic, immunophenotypic and cytogenetic characteristics of 241 cases of AML M0, including 58 cases in children. Immunophenotyping showed a heterogeneous phenotype, anti-myeloperoxidase being positive in only about half of the patients. The immature nature of the blasts in AML M0 was confirmed by the high incidence of expression of markers associated with hematopoietic progenitor cells, such as CD34, HLA DR and CD117. Cytogenetic data were available from 129 patients. A normal karyotype was found in only 24%. Most of the abnormalities were unbalanced and chromosomes 5, 7, 8 and 11 were the most frequently affected. Survival data were available from 152 treated patients (63%). The median over-

all survival for all patients was 10 months, being 20 months for children ($n = 36$), 10 months for a group of young adults ($n = 50$) and ($p = 0.09$). Karyotype was not a prognostic factor influencing survival. AML M0 shows the immunologic characteristics of early progenitor cells, but the expression of the different markers and cytogenetic abnormalities is heterogeneous. The prognosis is poor compared with that of other *de novo* AML and similar to that of AML with multilineage dysplasia or AML following myelodysplastic syndromes.

IMPROVEMENT OF THE CHRONIC LYMPHOCYTIC LEUKEMIA SCORING SYSTEM USING SEMI-QUANTIFICATION OF FLUORESCENT SIGNALS

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The diagnosis of B-cell lymphoproliferative disorders is based on multiparameter analysis including clinical features, cell morphology, immunologic markers, histopathology and molecular genetics. The scoring system developed by Matutes *et al.*,¹ based on the immunophenotypic analysis of five membrane markers (CD5, CD22, CD23, FMC7, Smlg), is helpful to distinguish chronic lymphocytic leukemia (CLL) from other B-cell lymphoproliferative diseases (non-CLL). However, this scoring system is not fully exploited due to the lack of standardization. The aim of the present study was to propose a standardized flow cytometry method allowing calibration of fluorescent signals and reproducible evaluation of antigen expression level on the lymphocyte surface. Double labeling was performed with antibodies directed against each of Matutes's score antigens combined to CD19 for gated analysis of the B-lymphocyte subpopulation. Autostandardization of the instrument using fluorescent calibrated beads (Flow Set, Beckman Coulter) allowed semi-quantitative and reproducible assessment of antigen expression level. Objective cut-off values of fluorescence intensity for score calculation were defined by performing immunophenotyping analysis of peripheral lymphocytes from 20 patients with CLL and 20 patients with non-CLL lymphoproliferative disorders (hairy cell leukemia, mantle cell lymphoma, splenic lymphoma with villous lymphocytes or prolymphocytic leukemia.). Application of this methodology to 50 patients with monoclonal B-cell lymphocytosis showed that score values correlated with cytological data and patients were correctly classified between having CLL or other B cell lymphoproliferative diseases. We concluded that the methodology proposed improves the contribution of Matutes's score in the diagnosis of B-lymphoproliferative disorders. Standardized semi-quantitative determination of antigen expression level may also provide useful information for identifying markers of prognosis and performing longitudinal monitoring of patients or multicenter studies.

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ROUTINE USE OF IMMUNOPHENOTYPING BY FLOW CYTOMETRY IN TISSUES WITH SUSPECTED HEMATOLOGIC MALIGNANCIES: ROLE IN DIAGNOSIS AND DETECTION OF DOUBLE PATHOLOGY

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Immunophenotype is an essential parameter in the diagnosis of hematologic malignancies. Flow cytometry (FC) is used in the analysis of bone marrow or peripheral blood samples but is less frequently used in the evaluation of tissue biopsies with suspected hematologic malignancies. The aim of this study was to analyze the role of FC of tissue samples obtained for the diagnosis of possible lymphoproliferative disorders. *Methods.* Consecutive biopsies (n=422) were studied using standard morphology, immunohistochemistry (IHC), and FC. Results of FC were obtained in less than three hours and were interpreted independently from morphology and IHC. *Results.* A strong correlation between malignant disease and abnormal pattern of FC was observed (218/250) except for Hodgkin's disease (HD) ($p < 0.001$). Overall, the negative predictive value was 0.79 and positive predictive value was 1. Light chain restriction was observed in 182/201 of B-cell lymphomas and in 0/142 non-B cell disorders by FC, whereas it was detected only in 31/121 B-cell lymphomas by IHC. The phenotypic profile of B-cell malignancies assigned by FC showed a good correlation with the final histologic diagnosis. All T-NHL showed an abnormal T-cell phenotype. In addition, FC allowed a rapid diagnosis of infrequent or high-grade malignancies (for instance histiocytic sarcoma, acute myeloid leukemia or T-lymphoblastic lymphoma). The combination of methodologies enabled the diagnosis of double pathology in 1% of patients (composite lymphoma and chronic lymphocytic leukemia and metastatic infiltration). *Conclusions.* FC is a fast and reliable methodology in phenotyping tissue samples, which easily detects infrequent hematologic malignancies and disease-specific phenotypes and light chain restriction in B-cell lymphomas. Moreover, the simultaneous recognition of different cell populations allows the diagnosis of composite cell lymphomas, or double pathologies.

CELLQUANT CALIBRATOR: A NEW FLEXIBLE TOOL FOR ROUTINE QUANTITATIVE IMMUNOPHENOTYPING

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Quantitative immunophenotyping of leukocytes has long been proposed to improve the flow cytometry-based classification of lymphoproliferative diseases. The advantage of measuring levels of expression of differentiation antigens is often

recognized. Recently, it has been again suggested in the differential diagnosis of B-cell chronic lymphoproliferative disorders.¹ However, access to appropriate tools is still limited. A new assay (CellQuant Calibrator kit, BioCytex, Marseille, F) is proposed: this assay is flexible enough to use each laboratory's panel of unlabeled IgG MAbs and counter-staining reagents. This no-wash indirect immunofluorescence (IF) method also fulfils the following requirements. The assay: (1) provides reproducible intra- and inter-laboratory quantitative data; (2) works on whole blood samples as well as on separated cells; (3) permit dual (or multiple) IF staining to focus measurements onto defined cell subset(s); (4) is applicable in routine use; (5) has a single calibration of the measuring fluorescence channel that is common to all MAbs of the laboratory panel; and (6) has a good enough dynamic range to measure both low levels (~1,000 sites/cell) as well as high levels (~300,000 sites/cell) of antigen expression. The potential of this new standardized assay will be illustrated with examples of: (1) differential quantitative phenotypes of *classical* B-CLL cases (Matutes score = 5) versus other B-cell lymphoproliferative disorders; (2) comparisons with the expression levels of the same antigens on normal adult peripheral blood B-lymphocytes; (3) measurements of putative prognostic markers (CD38 on B-CLL); (4) quantitative values of various surface antigens on T-cell subsets using dual and triple IF staining on normal and pathologic samples (viral infections, leukemia). Furthermore, multiple measurements made using different flow cytometers both internally and in external laboratories using stabilized blood samples will illustrate the repeatability and reproducibility of quantitative data and thus the potential for multicenter studies.

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TELOMERE LENGTH OF BLAST CELLS IN ACUTE MYELOBLASTIC LEUKEMIA DETERMINED BY FLOW CYTOMETRY

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Telomeres are repeated sequences located at the end of chromosomes and are essential for the stable maintenance of chromosomes because their length serves as a mitotic clock. The cell loses this sequence during each division which contributes to senescence and cell death. Telomerase is a specific DNA polymerase which maintains the telomere length and potentially leads to unlimited cell proliferation. It is active in germline cells and in the majority of cancers but normally inactive in most somatic cells. As high levels of telomerase, which may contribute to the capacity of proliferation of blast cells, were observed in some leukemias, we tried to measure the length of telomeres by flow cytometry using the *Dako Telomere PNA/FITC kit/Dako PNA probes for flow*. The relative telomere length (RTL) was calculated using the mean of fluorescence of cells of interest in G0/G1 phase compared to that of control cells. We studied peripheral white blood cells (WBC) from 8

healthy subjects and 15 cases of AML (7 cases of AML1 and 8 of AML5). In normal peripheral WBC, the mean RTL was 15.16 arbitrary units (UA) \pm 2.53 (max: 18.72; min: 11.91). In AML-1 the RTL was 8.09 \pm 3.4 UA (max: 12.5; min: 1.1). In AML-5 the RTL was 7.84 \pm 1.6 UA (max: 9.66; min: 4.21). We observed a smaller RTL in AML than in normal subjects contrasting with the high telomerase level generally reported enforcing the idea of a deregulation of this enzyme. This study is ongoing on larger series to determine the role of the RTL in the progression of acute leukemia.

FOUR INTRACELLULAR ENZYMES DETECTED BY FLOW CYTOMETRY IN NORMAL BONE MARROW CELL POPULATIONS SEPARATED BY CD45 EXPRESSION

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Flow cytometry is widely used to detect protein or glycoproteins antigens by direct or indirect immunofluorescent techniques with monoclonal antibodies. Human cells also contain proteolytic enzymes with specificity for a variety of peptide substrates. This cytochemical characteristic has been used for a long time in hematology in order to define the lineage commitment of blast cells i.e. the myeloperoxidase in myeloid blast cells. Cell Probe reagents are synthetic, non-fluorescent substrates which can be cleaved by these enzymes to form a fluorescent product (rhodamine 110). The resulting fluorescence from the hydrolysis of the Cell Probe substrate provides a measure of enzyme activity within each sample. As rhodamine 110 is excited at 468 to 509 nm and emits at 504-541 nm, analysis can be performed on an Argon-ion laser flow cytometer with a 525 nm filter (FL1). In order to determine their usefulness in clinical practice, we studied four of these reagents on normal bone marrow from 15 healthy subjects. The different cell populations were separated according to CD45 expression/SSC level: lymphocytes, monocytes, stem cells and granulocytes. Reagents sensitive to galactosidase, to cathepsin C and G, to non-specific esterases and to neutrophil elastase and pancreatic elastase were studied. Technically, the timetable must be very precise to allow good detection of an activity. The fluorescent ratios are indicated in the table below and although galactosidase activity was low or undetectable, other enzyme activities were quite substantial. Usually, these activities were present in all the populations of interest.

	Galactosidase	Cathepsine D	Esterase	Elastase
Stem cells	1.87 \pm 0.84	4.34 \pm 2.65	18.24 \pm 9.48	3.03 \pm 1.14
Monocytes	2.7 \pm 2.97	6.52 \pm 5.66	11.36 \pm 6.52	4.49 \pm 3.02
Granulocytes	2.01 \pm 1.28	11.95 \pm 10.56	20.35 \pm 16.3	15.71 \pm 18.2
Lymphocytes	1.98 \pm 1.8	4.7 \pm 4.37	8.15 \pm 4.73	1.73 \pm 0.72

EXPRESSION OF ANTIGEN CD34 AND CD71 ON HEMATOPOIETIC STEM CELLS. THERAPEUTIC INTEREST IN TROPICAL URBAN AREAS (ABIDJAN, COTE D'IVOIRE)

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Bone marrow transplantation is nowadays a frequent treatment used in cancer therapy and leukemia. Hematopoietic stem cells (HSC) exist not only in the bone marrow (BM) but are also found in umbilical cord blood (UCB) and in peripheral blood (PB). The antigen CD34 exists on stem cells and on committed progenitors. HSC in the G₀ quiescent state of the cell cycle do not express CD71, the transferrin receptor. In contrast, myeloid committed progenitors express CD71 but this antigen is not found on the mature blood cells. We applied two-color flow cytometric analysis using monoclonal antibodies CD34 and CD71. We studied 93 samples of mononuclear cells (MNC) from 31 BM, 31 UCB and 31 PB (without mobilization). The highest proportion of MNC was found in the BM (BM:8863 MNC/ μ L; UCB:5451/ μ L; PB 3034/ μ L; $p=0.0001$). The percentage of MNC CD34⁺ was nearly the same in the 3 sources (BM: 9.52%; UCB: 7.62%; PB: 6.84%; $p > 0.05$). BM and UCB contained more committed progenitors (CD 34⁺71⁺) than PB with respectively 64.5%, 63.1% and 17.8% ($p=0.0001$). In contrast, the MNC CD34⁺ of the PB contained more primitive progenitors CD34⁺71⁻ (82.2%) than BM (37%) and UCB (35.5%); ($p=0.0001$). The use of CD 34 and CD 71 showed that BM and UCB contain nearly the same percentage of committed progenitors and primitive precursors. Given that UCB is easier to obtain, we suggest that it could be the best solution for HSC transplantation in tropical urban areas.

SESSION B THERAPEUTICS

RECOMBINANT ANTIBODIES: TAILOR-MADE MOLECULAR PRECISION TOOLS

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Recent advances in gene technology have greatly facilitated the genetic manipulation, production, identification and conjugation of recombinant antibody fragments, making them tools for a plethora of applications in research, diagnosis and therapy. The antigen binding domains of valuable monoclonal antibodies can be rescued from the hybridoma cell lines and produced in heterologous systems for *hybridoma immortalization*. Easy genetic manipulation of recombinant antibodies has improved our knowledge about the structure and functional organization of immunoglobulins. Genetic fusion and recombinant expression has led to the development of a zoo of new heterologous fusion proteins, e.g. immunotoxins. New fascinating perspectives, too, have been opened up by developments to select specific monoclonal antibodies outside the human body. To do this, one first has to construct huge antibody gene libraries. This is usually achieved by PCR-amplification from B-lymphocyte cDNA. Alternatively, antibody genes can be constructed *in vitro* by gene synthesis using *random-*

ized wobble-primers, or a combination of both methods. To screen these antibody libraries that contain many millions of different clones, a selection system is required with an efficiency comparable to that of the immune system. This can be achieved by displaying antibodies on the surface of microorganisms containing the antibody's gene, in analogy to the expression of the IgM antigen receptor on the surface of unactivated B-lymphocytes. Examples of these prokaryotic organisms are filamentous bacteriophage (*phage display*) or bacteria. This surface display generates a particle which mediates a physical link between the antigen binding function and the antibody genes. Using the affinity to the antigen, the whole organism can be identified out of billions of other non-specific ones. Specific clones binding to an antigen can then be amplified and used to produce the antibody fragment in *E. coli* or other suitable organisms. These new screening procedures provide the power to select one out of more than 10^{10} different expression clones in less than 1 mL. This method creates the potential for generating human antibodies which could never be obtained from the blood stream. For example, antibodies to highly toxic substances or antigens which our immune system tolerates have been developed. By random or designed mutations, affinity or specificity of the antigen binding can be changed dramatically, reaching for example affinities in the femtomolar range which are never observed with natural antibodies. This process can be regarded as an *in vitro* imitation of somatic hypermutation during an immune response in our body. These methods open the way to a new chapter in the use of antibodies in research, diagnosis and therapy. For example, human antimouse immune response (HAMA), a major obstacle in patient treatment or *in vivo* diagnosis with conventional mouse monoclonal antibodies, has been successfully avoided. Furthermore, genetic coupling of the antibody to a heterologous protein by fusion of gene fragments generates new possibilities for immunotargeting. Immunotargeting utilizes the affinity of the antibody part of the fusion protein to increase the concentration/activity of the heterologous fusion part at sites where antigen is present. This can be used to construct immunotoxins or to generate bispecific and/or multifunctional proteins to retarget the T-cell response to tumor cells. A large variety of other fusions have been constructed and demonstrate the potential of antibody gene engineering for generating new therapeutic and diagnostic agents.

GENERATION OF POTENT Th1 RESPONSES FROM PATIENTS WITH LYMPHOID MALIGNANCIES AFTER DIFFERENTIATION OF B-LYMPHOCYTES INTO DENDRITIC-LIKE CELLS

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Dendritic cells (DCs) are a group of potent antigen-presenting cells (APC) specialized for initiating T-cell immune responses. They originate from the bone marrow, and upon stimulation with bacterial products, cytokines, or CD40 ligation, they

acquire the ability to migrate to the secondary lymphoid organs. *In vitro* DCs can be generated from human CD34⁺ bone marrow cells and CD14⁺ peripheral blood monocytes after culture with different cytokine combinations. Since most leukemic cells and tumors in general are devoid of APC capacities, various strategies have been used to increase their recognition and confer the capacity of antigen presentation on them. Because of our interest in the design of vaccine immunotherapy protocols for the adjuvant treatment of patients with lymphoid malignancies (LM), we chose to explore the capacity of human acute lymphoblastic leukemia (ALL), chronic lymphocytic leukemia (CLL), and plasma cell leukemia (PCL) to differentiate into cells with APC and DC features. Our results from a group of 10 patients demonstrate that such an approach is feasible. Leukemic cells could be induced in the presence of interleukin-4 and CD40L to exhibit a DC morphology with phenotype of mature DC-like cells. They could also induce a potent proliferative response in naive CD4⁺ T-cells. In addition, they expressed chemokine receptor CCR-7 and CD62L, and could drive T cells towards a Th1 response with secretion of IFN- α . Our strategy leading to increased LM cell immunogenicity may have potential clinical applications and LM appear to be attracting candidates for adjuvant vaccination and adoptive immunotherapy.

ALTERED HLA EXPRESSION IN NON-HODGKIN'S LYMPHOMA IS FREQUENTLY ASSOCIATED WITH AGGRESSIVE SUBTYPE, RELAPSE OR TRANSFORMATION

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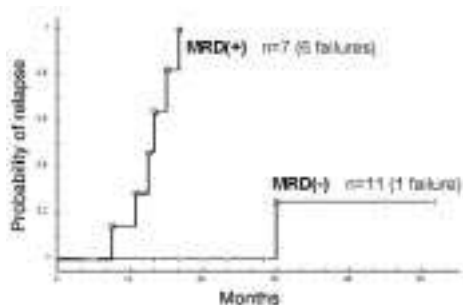
HLA molecules play a major role in the recognition of human cells which is a key step in anti-tumoral response. HLA loss is observed in different malignancies, inducing escape from immune surveillance. HLA-G, a non-classical class Ib molecule, is currently considered to be an immune tolerance-inducing molecule. So its expression in cancer could be relevant for immune escape. Since few data are available in non-Hodgkin's lymphoma (NHL), HLA molecules were prospectively studied in 614 cases using flow-cytometry (FCM) with specific anti-HLA class I monoclonal antibody (MoAb; clone W6/32) and anti HLA class II MoAb (B8.12.2; HLA-DR). Furthermore, a specific anti-HLA-G MoAb (87g) was tested in 50 cases, including 20 cases selected for their defective HLA class I expression. In 64 cases (10.4%), lymphomatous cells exhibited a lower W6/32 mean fluorescence intensity (MFI) compared to reactive cells (multicolor direct immuno-fluorescence assay using FITC, anti-CD2 MoAb, PE W6/32 MoAb, PE-Cy5 CD19 MoAb). The characteristics of these cases were i) the diversity of histologic entities (FL (20%); DLCL (56%); MCL; Burkitt, NK/T lymphoma (6%); ii) the frequency of relapse or transformation (34%) iii) the high incidence in high grade NHL (22.9%) compared to low grade NHL (6.4%), iv) the severity of the class I defect in 50% of the cases and mainly in high grade NHL. The HLA-DR defect was always associated with severe class I defect (12 cases; 2%). HLA-G protein was only expressed in three class I defective cases with normal HLA-DR expression. Abnormalities of HLA molecules could be observed in NHL similarly to in other

malignancies. FCM was particularly useful for this type of quantitative evaluation. These HLA alterations frequently appeared as a second event at relapse or at transformation suggesting a role in lymphomagenesis.

DIFFERENT SIGNIFICANCE OF MINIMAL RESIDUAL DISEASE AFTER AUTOLOGOUS AND ALLOGENEIC STEM CELL TRANSPLANTATION FOR CHRONIC LYMPHOCYTIC LEUKEMIA: PROGNOSTIC AND THERAPEUTIC IMPLICATIONS

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The clinical significance of minimal residual disease (MRD) in chronic lymphocytic leukemia (CLL) patients receiving stem-cell transplants (SCT) has not been extensively studied. We investigated MRD in peripheral blood and bone marrow samples in 32 consecutive patients (21 M/11 F; median age, 47-yr; range: 29-61) undergoing transplants (14 allo-SCT, median follow up 40 months, range: 6-120, and 18 auto-SCT, median follow up 25 months, range: 5-62) for poor-risk CLL. Methods included flow cytometry using triple-color combinations of CD20/CD5/CD19; CD22/CD23/CD19; and light chain κ/λ /CD19; and PCR of the CDRIII region, based on consensus primers. **Results.** MRD persisted in 7/18 autografted patients (39%) with a constant increase of MRD. A clinical relapse was observed in 6 of them. MRD reappeared in three autografted patients with one presenting a clinical relapse (see figure). MRD status after autograft anticipates clinical progression in most patients with an actuarial risk of relapse of 87 % (± 12) at 25 months of MRD detection.



In allografted patients, MRD was detected in 5/11 responding patients who had survived more than three months (45%). Low levels of MRD were intermittently detected in two cases and persistently in two, and a delayed clearance was observed in one. None of the MRD+ patients after allo-SCT has relapsed. In conclusion, whereas the presence of MRD after auto-SCT in CLL patients is highly predictive of clinical relapse, MRD positivity after allo-SCT is not necessarily followed by clinical progression, a fact most likely related to the graft-versus leukemia effect.

SESSION C ACUTE LEUKEMIA AND APOPTOSIS

FUNCTIONAL FEATURES AND TREATMENT OUTCOME OF T-LINEAGE ACUTE LYMPHOCYTIC LEUKEMIA

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Precursor T-cell acute lymphocytic leukemia (ALL) results from clonal expansion of hematopoietic progenitors that have undergone leukemic transformation at distinct stages of T-cell differentiation and, similar to precursor B-cell ALL, is manifested by a strong interpatient cell-biologic heterogeneity. Various immunophenotypic features have been associated with an increased risk of treatment failure, including an immature pro-/pre-T-ALL phenotype, membrane expression of CD3 or MHC class II antigen, and negativity of CD2, CD5, THY antigen (similar to CD1), or CD10. The prognostic impact of these factors, however, has differed according to the treatment strategies used, and immunophenotyping still represents a controversial prognostic factor that has not been routinely used for risk classification or assignment to novel treatment strategies in high-risk precursor T-cell ALL patients. More recently, our own results and other studies have convincingly shown that children and adults with pre-T ALL differ markedly with respect to their phenotypic and genotypic features, suggesting an arrest of adult pro-/pre-T ALL at a less mature differentiation stage than in childhood, which may be closely related to the worse treatment outcome observed in these patients. Accordingly, leukemic blasts from patients with pre-T ALL were capable of multilineage differentiation *in vitro* both spontaneously and after stimulation with appropriate cytokines, demonstrating that acute leukemia in these patients evolved from *in vivo* transformation of immature pluripotent hematopoietic cells. Additionally, the ALL-BFM trials and, more recently, two other multicenter trials in childhood ALL, using similar maturational staging systems, have lent strong support to evidence that children with cortical (CD1a-) precursor T-cell ALL have a better early response to treatment, as illustrated, for instance, by the *in vivo* response to corticosteroids, and a significantly longer duration of event-free survival (EFS) than those with an immature or mature precursor T-cell phenotype. Similar data, i.e., a significant improvement in survival of adult patients with CD1, CD2, CD4, and CD5 expression compared with patients not expressing these antigens, have been described by the Cancer and Leukemia Group (CALGB) and the German Multicenter Study Group for Adult ALL (GMALL). Given these clinically relevant correlations between immunophenotypic features and treatment outcome in precursor T-cell ALL, we have recently focused on the investigation of apoptosis-related parameters, including spontaneous apoptosis *in vitro* and modulation of apoptosis by interleukin-7 (IL-7). This cytokine plays a non-redundant role as an anti-apoptotic factor in normal T-cell development by regulating bcl-2 expression in immature thymocytes and mature T-cells. Our studies suggested that maturational stages of precursor T-cell ALL may differ as to their accessibility to apoptotic programs, with lymphoblasts express-

ing CD1a or exhibiting a selection-related phenotype being more susceptible to apoptosis than leukemic lymphoblasts with an immature phenotype. In a series of childhood precursor T-cell ALL (N=81), CD1a positive cases showed an increased *in vitro* susceptibility to dexamethasone- as well as to doxorubicin-induced apoptosis as compared with pro-/pre- and mature T-ALL ($p < 0.05$). Apoptosis-related parameters (e.g., Bax, Bcl-2, CD95 and CD95-induced apoptosis) did not account for differential susceptibility to drug-induced apoptosis. Analysis of clinical data showed that *in vitro* susceptibility to dexamethasone as well as cytokine (IL-7) responsiveness closely correlated with an early *in vivo* therapy response as determined by percentages of blast cells in bone marrow on day 15 ($p < 0.01$). EFS of childhood precursor T-cell ALL according to maturational stages and corresponding functional features will be presented in detail.

INVOLVEMENT OF CASPASES IN TERMINAL CELL DIFFERENTIATION

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Most of the studies concerning caspases, a family of cysteine proteases, have highlighted the relationship between their activation and the occurrence of cell death by apoptosis. However, caspase-1 was initially identified as the protease responsible for maturation of the multifunctional cytokine IL-1 β and was subsequently demonstrated to trigger the maturation of IL-18. Caspase-3 activation may play a role in the maturation of IL-16 in non-apoptotic T-cells. In addition, caspases are involved in T-cell activation, in cell cycle regulation and in various differentiation pathways. Erythropoiesis was proposed to be regulated by a negative feedback loop in which mature erythroblasts expressing death-receptor ligands inhibit the differentiation of immature erythroblasts through engagement of their death receptors and caspase-mediated degradation of the transcription factor GATA-1. We have identified a distinct process in which a transient activation of caspases that does not lead to GATA-1 cleavage is required for erythroid differentiation. Caspase activation was also demonstrated to be required for the terminal differentiation of lens epithelial cells and keratinocytes. In these cell types, terminal differentiation is associated with enucleation that has been regarded as a caspase-mediated incomplete apoptotic process. We have observed that caspase-3 is also transiently activated in U937 human leukemic cells undergoing phorbol ester-induced differentiation, a process that does not end with enucleation. Some mechanisms that could account for caspase activity regulation in leukemic cells undergoing differentiation will be presented and the potential role of caspase deregulation in diseases associating caspase activation and abnormal differentiation will be discussed.

CRYPTIC t(5;14)(q35;q32) TRANSLOCATION IN CHILDREN WITH T-CELL ACUTE LYMPHOCYTIC LEUKEMIA: IMMUNOLOGIC FEATURES

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We have recently identified, by an IPM-FISH technique (IRS-PCR multiplex FISH), a new t(5; 14)(q35; q32) cryptic translo-

cation in children with T-ALL.¹ The detection of this abnormality, totally cryptic by conventional cytogenetic analysis (R-banding), was confirmed by whole chromosome 5 and 14 painting probes. Simultaneously, Bernard *et al.* showed at the molecular level that t(5; 14)(q35; q32) leads to over-expression of the Hox11L2 gene. Over-expression of this developmental gene seems to be restricted to only T-ALL with t(5; 14). From a limited number of patients, t(5;14) frequency was estimated to be about 20-30 %. Considering the potential impact of this abnormality, we were interested in looking for the existence of peculiar immunologic features in these proliferations. We report here three cases of t(5; 14)(q35; q32) T-ALL out of 10 in our series, detected by FISH and expressing HOX11L2 by RT-PCR. By flow cytometry, the blast immunophenotyping showed the existence of common characteristics between our three patients: expression of the CD1a, CD2, CD4, CD5, CD7, and CD8. Due to the positivity of the cortico-thymocyte CD1a marker, all these proliferations were classified as being T-III (according to the EGIL classification). Alongside this common denominator, immunologic study also showed a part of the heterogeneity in phenotypes: no expression of Tdt for a patient, presence of a myeloid marker, positivity of the CD10 (2/3 patients), expression of a TCR α in one proliferation. The 5 patients reported by Bernard *et al.* also belong to the T-III group (EGIL). Despite this visible agreement between the T-III phenotype and the presence of t(5; 14), (the only cytogenetic abnormality found in all cases listed today), immunophenotyping also showed numerous differences, so the interpretation of our results can be envisaged only within the framework of a larger series. In conclusion, t(5; 14)(q35; q32) is a recurring abnormality probably frequent in children with T-ALL; there are now cytogenetic and molecular means to reach a diagnosis routinely: these tools should quickly allow retrospective and forward-looking analyses which will serve as a basis to clarify immunologic data of this new entity.

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EXPRESSION OF BCL-2 FAMILY PROTEINS AND CASPASE ACTIVITIES IN MYELODYSPLASTIC SYNDROMES

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Introduction. It has been recently demonstrated that an excess of apoptosis is the cause of marrow failure in myelodysplastic syndromes (MDS), particularly in *early MDS* refractory anemia (RA) and RA with ringed sideroblasts (RAS) FAB-subtypes as compared to *late MDS* RA with excess of blasts (RAEB), RAEB in transformation (RAEB-T) and chronic myelomonocytic leukemia (CMML). To investigate the role of caspase-3 and Bcl-2-related proteins in the increased apoptosis in the initial to the late stages of the disease, we studied in bone marrow mononuclear cells (BMMNC) the expression of some Bcl-2 family proteins (anti-apoptotic members Bcl-2, Bcl-xL and pro-apoptotic Bax, Bad, Bak, Bcl-xS), and the expression and activity of caspase-3. The elucidation of apoptotic pathways in hematopoietic cells may be important for the understanding of

molecular events involved in MDS pathogenesis and its leukemic progression. In order to analyze the contribution of cell death receptors and mitochondrial pathways in MDS apoptosis, we measured caspase-8 and -9 activities. Moreover we studied the expression of the pro-apoptotic-Bcl-2-member Bid, and release of cytochrome-c by immunoblotting. Finally, to determine whether the Fas receptor-Fas ligand (FasR /FasL) system is implicated in the induction of MDS apoptosis, we performed all these experiments with or without an anti-FasR antibody clone ZB4. **Materials and Methods.** The intracellular expression of Bcl-2-related proteins and caspase-3 was assessed by flow cytometry (FCM). Caspase-3, -8 and -9 activities were measured by a fluorimetric assay, and a Western blot analysis confirmed the expression of caspase-3 including the cleaved (activated)-p17 fragment. For each experiment controls were performed on samples of normal bone marrows. **Results.** The percentage of BMMNC expressing Bcl-2 and Bcl-xL was significantly higher in RAEB, RAEB-T and CMML than in RA and RAS ($p < 10^{-4}$). Conversely pro-apoptotic proteins Bad, Bak, Bcl-xS were detected in a higher percentage of cells in RA and RAS ($p < 10^0$). RA and RAS were also associated with a significant increase of pro-versus anti-apoptotic ratio Bcl-xS/Bcl-xL compared to normal marrows or RAEB/RAEB-T. Also, caspase-3 expression in FCM was detected in a higher percentage of RA/RAS. Immunoblotting confirmed the expression of caspase-3, with the presence of the active p17 form in several cases of early MDS, generally associated with increased caspase-3 activity. The first results for apoptotic pathways seems to show the Fas-FaL interaction does not contribute to increased MDS apoptosis. **Discussion/Conclusions.** We describe here the association of pro-apoptotic Bcl-2-related proteins with early MDS, and the increase of anti-apoptotic proteins in late MDS associated with a lower Bcl-xS/Bcl-xL ratio and a poor outcome (survival and time to transformation). Moreover, we also demonstrate the involvement of caspase-3 in the apoptotic process and probably the participation of the mitochondrial apoptotic pathway. In conclusion, our data confirm that the control of apoptosis is deregulated in MDS cells.

EVALUATION ON U937 CELLS OF THE PRO-APOPTOTIC POTENCY OF DIFFERENT OXYSTEROLS AND OF THEIR ABILITY TO INDUCE INFLAMMATORY PROCESSES

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Oxysterols, are 27-carbon derivatives of cholesterol that contain additional oxygen atoms either on the steroid nucleus or on the side chain. Oxysterols possess diverse biological activities, and some of them are strongly cytotoxic to both normal and tumor cells. In the present study, U937 promocytic human leukemia cells were cultured in the presence of oxysterols (7 α -, 7 β -hydroxycholesterol, 7-ketocholesterol, 25-hydroxycholesterol, 19-hydroxycholesterol, cholesterol 5 α , 6 α -epoxide, cholesterol 5 β , 6 β -epoxide, 22R-hydroxycholesterol, 22S-hydroxycholesterol, 16 β -hydroxy-5 α -cholestane-3,6-dione) used in a range of concentrations from 5 to 80 μ g/mL for

6 to 24 h, and the ability of these compounds to induce apoptosis, and to stimulate IL-8 secretion and superoxide anions production was investigated. On U937 cells, no cytotoxic effects (evaluated by inhibition of cell growth, enhanced permeability to propidium iodide, loss of transmembrane mitochondrial potential, phosphatidylserine externalization) were found with 7 α -hydroxycholesterol, 25-hydroxycholesterol, 19-hydroxycholesterol, cholesterol 5 α , 6 α -epoxide, 22R-hydroxycholesterol, 22S-hydroxycholesterol, and 16 β -hydroxy-5 α -cholestane-3,6-dione (an oxysterol isolated from the red alga *Jania rubens*). However, IL-8 secretion quantified by ELISA was strongly stimulated with 25-hydroxycholesterol, and superoxide anions production (measured by flow cytometry with hydroethidine) was increased. Notheworthy, 7 β -hydroxycholesterol, 7-ketocholesterol, and cholesterol 5 β , 6 β -epoxide which were potent inducers of apoptosis also enhanced superoxide anion production but did not stimulate IL-8 secretion. Taken together, these data underline that oxysterols constitute useful tools for defining the relationships between apoptosis, cytokine secretion, and radical oxygen species production.

IMMUNOLOGIC PHENOTYPE OF CHILDHOOD ACUTE LYMPHOBLASTIC LEUKEMIA: A STUDY OF 49 CASES

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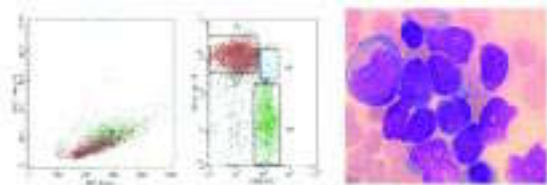
Immunologic phenotype determination is essential to the diagnosis and prognosis of acute lymphoblastic leukemia (ALL) in children. In this study we analyzed blood samples from 49 children with ALL (33 boys and 16 girls aged from 2 to 15 years; mean: 7 years). We classified these ALL as low risk in 18 cases, intermediate risk in 9 cases and high risk in 22 cases. Mediastinal enlargement was noted in 10 cases. Leukocytosis exceeding 50×10^9 cells/L was present in 20 cases. L1 type was noted in 42 cases and L2 in 7 cases. Cytogenetic study was done in 40 cases and showed an abnormality in 10 cases. The phenotype was B in 32 cases (65%) and T in 12 cases (25%). Myeloid markers were present in 2 cases. The phenotype was not possible to evaluate in 5 cases. The T phenotype was correlated to bulky forms with mediastinal enlargement. In conclusion, this study shows that immunophenotyping by flow cytometry is a valuable complement to reclassifying many forms of childhood ALL.

A BICLONAL ACUTE LEUKEMIA WITH A t(4;11) (q21;q23) TRANSLOCATION

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Childhood acute lymphoblastic leukemia (ALL) characterized by a t(4;11) (q21;q23) translocation occurs more frequently in younger infants and is marked by a bad prognosis. These ALLs are often associated with a high level of leukocytosis, hepatomegaly and frequently involve the brain and the spinal fluid. The sex ratio is unbalanced towards females. The blast cells normally contain fractions with either a lymphoid mor-

phology or a myeloid morphology. This suggests derivation from an early progenitor. The phenotype of blastic cells is generally early immature: most often proB (CD19⁺, TdT⁺, CD10⁻) with the concomitant expression of myeloid markers (CD15⁺, CD65⁺). Rare cases of acute myeloid leukemia (frequently myelomonocytic AML4 according to the FAB classification) have been reported (3% of AL with t(4;11)). We report here a case of an unusual AL with the translocation in which the two cell types show the morphologic, cytochemical and immunologic characteristics of lymphoid or monocytic blasts (biclinal). *Case report.* A two-month old infant presented with hepatosplenomegaly. The blood count showed a WBC 680 ×10⁹/L, hemoglobin 4.2 g/dL, platelets 24 ×10⁹/L, blasts: 100%. Bone marrow examination revealed 85% blastic cells with two morphologic features: 60 % of them were small and had a high nucleo-cytoplasmic ratio classified as lymphoblastic cells; 40% of them were large with a cytoplasm showing light granular aspects classified as monoblastic cells. *Cytochemistry:* peroxidase staining was negative in the small blasts and lightly positive in the large blasts. Butyrate esterase staining was negative in both populations. *The spinal fluid* was infiltrated (WBC: 6×10⁹/L) with monoblasts (83%) and lymphoblasts (13%). *The immunophenotype* of the bone marrow confirmed the presence of two blastic populations: *Pro B lymphoblastic cells* (EGILL classification) with the following phenotype CD19⁺, CD20⁻, CD10⁻, CD22⁺, CD24⁺, CD79a⁺, cμ⁻. *Blasts with monocytic phenotype:* CD33⁺, CD14⁺, CD15⁺, CD65⁺. *The karyotype* showed a translocation involving chromosomes 4 and 11 in t(4;11) (q21; q23) AML1-AF4 gene rearrangement was detected by RT-PCR. The TCRδ (Vδ2 - Dδ3) and IgH (FrIII - JH) loci were rearranged. Retrospectively, the fusion transcript MLL-AF4 was also found in the cord blood. The cord blood count was normal at this time. A complete remission, which has persisted for 3 months of follow-up, was obtained using the INTERFANT protocol. *Discussion.* We have reported here a typical case of infant acute leukemia with t(4;11) (q21;q23) translocation involving the MLL-AF4 rearranged gene. Two aspects of this case are striking: (1) *the presence of the clonal cells at birth* (retrospective analyses showed the transcript in the cord blood), and (2) the blasts cells were made up of a *biclinal population* and not a biphenotypic one: i.e. proB lymphoblasts and monoblasts (see figure below):



Immunosquattergram showing lymphoblastic B cells CD19⁺ CD33⁻ (R1) and monoblasts CD19⁻ CD33⁺ (R2). A few blasts expressed both markers, CD19 and CD33 (R3). The two populations are marked by morphologic features (right).

EXPRESSION OF MOLECULES OF THE TETRASPAN FAMILY ON BLAST CELLS FROM ACUTE LEUKEMIA PATIENTS

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The tetraspan family is a peculiar family of integral transmembrane proteins with 4 hydrophobic domains resulting in 2 extracellular loops and intracytoplasmic NH₂ and COOH termini. These molecules are involved in cell differentiation, adhesion and activation. They tend to belong to microdomains including integrins and/or MHC class II antigens. CD9 is one of the first tetraspans identified, initially on platelets and later on leukemic cells of the B lineage. TALLA-1 (CD231) is another tetraspan observed on some T-lineage ALL. Little is known, however, about the expression of other tetraspan molecules on leukemic cells. Here we report on a systematic prospective study of the expression of 11 tetraspan molecules on 52 acute myeloblastic leukemias, 16 acute lymphoblastic leukemias and 18 chronic lymphoproliferative disorders. Five tetraspans appeared frequently expressed. The most ubiquitous was CD82 (95% of the cases tested), followed by CD151 (59%), CD53 (44%), CD37 (43%) and CD9 (42%). All assessments were performed with the same protocol used to appreciate the fluorescence intensity of calibrated microparticles (Immunobrite, Beckman Coulter) thus allowing the quantitative expression of the tetraspans to be compared between patients. The large dispersion of fluorescence intensities observed was correlated to other immunophenotypic features.

IN SITU IDENTIFICATION OF OXIDATIVE DNA DAMAGE IN U937 CELLS TREATED BY VARIOUS PRO-APOPTOTIC COMPOUNDS USING IMMUNOFLOUORESCENT DETECTION OF 8-OXOGUANINE BY FLOW CYTOMETRY

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To determine the relationships between apoptosis and oxidative stress, the ability of 7b-hydroxycholesterol (50 μM) and 7-ketocholesterol (100 μM) to induce apoptosis and to stimulate superoxide anions production was compared with that of different pro-apoptotic antitumoral drugs (etoposide (50 μM), cycloheximide (200 μM)). All compounds induced apoptosis characterized by the occurrence of cells with fragmented and/or condensed nuclei, caspase-3 activation, PARP degradation, and internucleosomal DNA fragmentation. Only 7β-hydroxycholesterol, 7-ketocholesterol, and cycloheximide enhanced superoxide anion production measured by flow cytometry with hydroethidine as well as lipid peroxidation quantified by fluorimetry with thiobarbituric acid. Since 7β-hydroxycholesterol, 7-ketocholesterol-, and cycloheximide-induced apoptosis was associated with overproduction of radical oxygen species, we wondered whether these different pro-apoptotic molecules

were capable of inducing DNA damage. Analysis of microsatellite sequences at 12 loci did not show any instability. However, since the presence of 8-oxoguanine is considered as a marker of DNA damage resulting from oxidative stress, *in situ* detection of 8-oxoguanine was performed with the Biotrin OxyDNA Assay which uses a direct fluorescence technique. A FITC-conjugate binds to the 8-oxoguanine moiety of 8-oxoguanosine in damaged cells and fluorescence is detected by flow cytometry. In those conditions, 8-oxoguanine was identified in 7 β -hydroxycholesterol-, 7-ketocholesterol-, and cycloheximide-treated cells but not in untreated cells. Comparatively to conventional biochemical methods used to reveal 8-oxoguanine (liquid chromatography/mass spectrometry requiring cell lysis and subsequent purification of DNA) the Biotrin OxyDNA Assay looks very promising for evaluation of pathologic oxidative DNA damage in cell suspension.

SESSION D ACUTE MYELOID LEUKEMIA

IMMUNOPHENOTYPIC CLUSTERING OF MYELODYSPLASTIC SYNDROMES

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Myelodysplastic syndromes (MDS) constitute a group of heterogeneous diseases of a bone marrow precursor for which immunophenotypic characterization is still irrelevant in spite of the accuracy and the sensitivity of the flow cytometry technique. The aim of this study was to determine whether immunophenotypic abnormalities could be defined in MDS and could correlate with the FAB classification and cytogenetics. Analysis was performed on 275 bone marrow samples (207 patients with MDS, 68 controls) and 25 control blood samples. Immunophenotyping was based on a primary gating of blast cells, monocytes and granulocytes for CD45 antigen expression and side scatter light diffraction. For each marker, the result was expressed as the ratio between the mean of the fluorescence of the specific signal and the mean of autofluorescence. Phenotypic hierarchical clustering was performed to analyze the results. These results shows that (i) phenotypic clustering partly discriminates patients with RAEB/RAEB-T, CMML and RA/RARS for CD45w blast cells and patients with RA/CMML, RARS and RAEB/RAEB-T for granulocytes, (ii) the most discriminating markers were CD16, CD34, CD36, CD38, CD71 and HLA-DR for blast cells and CD11b, CD13, CD33, CD36 CD38, CD71 and HLA-DR for granulocytes, (iii) percentage of blasts clustered with high levels of CD34 expression on these cells, with a poor prognosis, (iv) high levels of CD36 expression defined a cluster of poor prognosis on both blast cells and granulocytes and (v) high levels of CD71 expression on granulocytes discriminate RARS from other MDS categories. These results fully support a close relationship between phenotypic abnormalities and bone marrow dysplasia.

IMMUNOPHENOTYPIC ANALYSIS OF MINIMAL RESIDUAL DISEASE IN HEMATOLOGIC MALIGNANCIES: FROM TECHNIQUES TO CLINICAL UTILITY

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In spite of the fact that for a long time immature leukemic cells have been believed to reflect the immunophenotypic characteristics of their normal counterparts, at present it is well-established that this is true only to a certain extent. Accordingly, recent studies based on the flow cytometric analysis of three and four color multiparametric stainings have shown that neoplastic cells from acute leukemia patients display aberrant phenotypic features with a frequency as higher as > 95% among precursor B-acute lymphoblastic leukemia (ALL), 100% in T-ALL and > 75% in acute myeloblastic leukemia (AML). While in precursor B-ALL and T-ALL aberrant expression of

myeloid-associated markers (CD13, CD15, CD33, CD65 and CD66) on lymphoblasts is relatively frequent ($\leq 75\%$ and 60% , respectively) the incidence of cross-lineage antigen expression in AML is much less frequent ($\leq 20\%$). The presence of antigen overexpression (mainly CD10 and CD34) is almost exclusive to precursor B-ALL, while almost undetectable in T-ALL (0%) and present in only a small proportion of AML ($\leq 10\%$). By contrast, detection of ectopic phenotypes in the bone marrow is a frequent finding in T-ALL ($\leq 70\%$) as compared to in precursor B-ALL (0%) and to a less extent AML ($\leq 30\%$). Finally the presence of asynchronous antigen expression is a common finding in all disease groups, its incidence ranging from 40% to 80% of the cases. Such an incidence of aberrant phenotypes allows the detection of more than one aberrancy in most cases of acute leukemia. This facilitates the use of more than one patient-built specific phenotypic probe to follow MRD once the patient has achieved morphologic complete remission. This is particularly important since it has been shown that phenotypic changes, even involving aberrant phenotypes, may occur in up to 16% of all AML cases which highlights the need to follow all aberrant phenotypes detected at diagnosis, once morphological complete remission has been achieved in order to avoid false negative results. Dilutional experiments have shown that the sensitivity of flow cytometry immunophenotyping for the detection of minimal numbers of residual leukemic cells depends on the type of phenotypic aberrancies and ranges from 10^{-4} to 10^{-6} . From the clinical point of view, persistence or a gradual increase in the number of residual leukemic cells has been associated in ALL with a higher incidence of relapses and a shorter survival. In a similar way, in AML the persistence of relatively *high* levels of minimal residual disease in the first bone marrow sample in morphologic complete remission obtained after induction therapy is the most powerful independent prognostic factor for predicting disease-free survival.

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A CONSENSUS IMMUNOPHENOTYPIC PANEL FOR LEUKEMIA AND OTHER HEMATOPOIETIC PROLIFERATIONS

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Flow cytometric immunophenotyping of leukemias and lymphomas has become widely accepted in the complete diagnostic evaluation of patients with hematolymphoid neoplasms. This diagnostic modality both contributes to diagnostic accuracy and provides a basis for subsequent therapeutic monitoring for minimal residual disease. As with other diagnostic testing methods interlaboratory standardization is an important issue for reasons of both quality control and diagnostic consistency. As the number of available monoclonal antibodies is constantly increasing and the diagnostic experience of laboratories is variable, the Clinical Cytometry Society (CCS) has sought to facilitate consensus on the optimal use of monoclonal antibodies in the clinical practice of flow cytometric immunophenotyping. Recent surveys of the CCS membership (*Cytometry* 2001; 46:177-83) and international consensus meetings sponsored by CCS (*Cytometry* 2001; 46:23-7) have provided information regarding current practice of clinical cytometry in the evaluation of leukemia, lymphoma, and related hematopoietic neoplasms. Although absolute agreement on the optimal antibody panel was not achieved, guidelines and recommendations for acceptable diagnostic practice were defined. For instance there was general agreement regarding the need for more than one B cell, T cell, and myeloid markers in the evaluation of hematolymphoid neoplasms. Adequate evaluation of chronic lymphoproliferative disorder and lymphoma should include a minimum of 12-16 monoclonal reagents. Plasma cell disorder evaluation requires a minimum of 5-8 markers and acute leukemia evaluation requires at least 20-24 monoclonal antibodies. It is important that such international dialogue continues as diagnostic advances in the phenotypic characterization of hematolymphoid neoplasms are developed. Economic pressures to minimize the use of monoclonal reagents need to be balanced against compromise of diagnostic accuracy and the benefits of sensitive therapeutic monitoring. This presentation will summarize the consensus findings to date as developed by CCS activities and advocate the ongoing need for similar such international cooperation in diagnostic cytometry.

IMMUNOLOGIC CLASSIFICATION OF ACUTE MYELOBLASTIC LEUKEMIAS

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The immunologic analysis of a limited panel of surface or intra-cellular antigens allows most cases of acute leukemia to be assigned to a specific lineage. Immunophenotyping of blast cells has thus become an elective and useful tool for identifying acute myeloblastic leukemia (AML). However, because of

the large heterogeneity of myeloid related lineages, no clinically relevant immunologic classification of AML has been devised so far. In order to attempt to set up such a classification, we analyzed the relative distribution and expression of eleven differentiation antigens (CD7, CD13, CD14, CD15, CD33, CD34, CD35, CD36, CD65, CD117, HLA-DR) in a test series of 176 consecutive AML cases. Statistical tools of clustering were used to remove antigens with overlapping distribution, leading to the proposal of a classification with five AML subsets (MA, MB, MC, MD, ME) based on the expression of four groups of seven antigens (CD13 or CD33 or CD117, CD7, CD35 or CD36, and CD15) (table below).

	CD13 or CD33 or CD117	CD7	CD35 or CD36	CD15	Test series	Test+ training series		
MA	+	-	-	-	33	19	179	20
MB	+	+	-	-	10	6	52	6
MC	+	±	+	-	44	25	180	20
MD	+	±	±	+	84	48	454	50
ME	-	±	±	±	5	3	44	5
Total					176		909	

This stratification, validated in a second AML cohort of 733 patients (training series), has no overlap with the FAB classification. MA and MB AML have exclusively myeloid features with only seldom extramedullary disease. The expression of lymphoid or non-lineage antigens is rare in MA AML. No cases of acute promyelocytic leukemia (APL) were observed within MB AML. The cytologic features of MC AML are either myeloid (81%), CD7 expression being related to the expression of B lymphoid lineage antigens, or erythroblastic (11%). MD AML more frequently have a high WBC than other subsets. A high leukocyte count is related to the expression of CD35 or CD36 and CD14 and to monoblastic differentiation. ME AML lack CD13, CD33 and CD117 but display other signs of myeloid differentiation including MPO expression (61%). The outcome was unfavorable in patients over 60 years old or with WBC higher than $30 \times 10^9/L$. However specific independent prognostic factors appeared to be related to poor overall survival in specific immunologic subsets. These were CD34 expression ($p < 3 \times 10^{-4}$) in MA AML, CD7 positivity in MB AML, non-APL cases ($p < 0.03$) in MC AML, CD34 positivity ($p < 0.002$) and CD14 positivity ($p < 0.03$) in MD AML, CD14 expression in ME AML ($p < 0.01$). In summary, the immunologic classification of AML that we propose, based on the expression of seven antigens (CD13, CD33, CD117, CD7, CD35, CD36, and CD15), and validated on a large cohort of patients, allows easy identification of 5 clinically relevant AML subsets with specific differentiation characteristics. In each classification subset, high risk AML patients can be identified, requiring the additional analysis of CD14 and CD34. These patients should be considered for alternative therapeutic strategies.

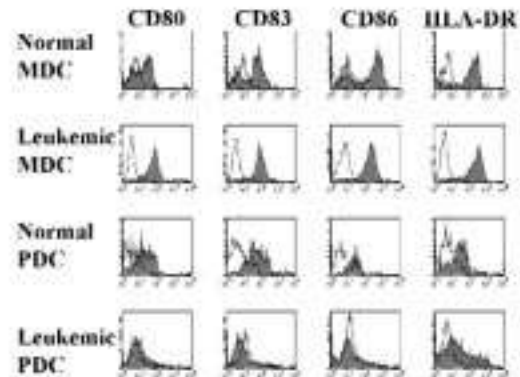
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CIRCULATING BLOOD DENDRITIC CELLS FROM MYELOID LEUKEMIA PATIENTS DISPLAY QUANTITATIVE AND CYTOGENETIC ABNORMALITIES AS WELL AS FUNCTIONAL IMPAIRMENT

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Dendritic cells (DCs) are responsible for the initiation of immune responses. Two distinct subsets of blood DCs have been characterized thus far. Myeloid DCs (MDC) and plasmacytoid monocytes (PDC) were shown to be able to promote polarization of naive T cells. In this study, based on immunophenotypic analysis, we show a dramatic quantitative imbalance in both circulating blood DC subsets in 37 patients with acute myeloid leukemias. Eleven patients (30%) displayed a normal quantitative profile, whereas 22 (59%) showed a tremendous expansion of either MDC, PDC or both subsets ranging from 0.04 to 41% for MDC and 0.22 to 14% for PDC. Finally, in 4 patients (11%), no DC subsets were detectable. Both MDC and PDC subsets exhibited the original leukemic chromosomal abnormality. *Ex vivo*, leukemic PDC but not leukemic MDC, have impaired capacity for maturation, decreased allostimulatory activity. Also, leukemic PDC are altered in their ability to secrete IFN- α . These data provide evidence that DC subsets *in vivo* may be affected by leukemogenesis and may contribute to leukemia escape from immune control.



Maturation capacities of MDC and PDC isolated from patients after *in vitro* culture. The two DC subsets MDC and PDC were sorted from the blood of healthy individuals or from leukemic patients and analyzed by flow cytometry after 72 hours of culture with either GM-CSF, IL-4 and CD40L (MDC) or IL-3 and CD40L (PDC) for their expression of DC marker CD83 and co-stimulatory molecules. Open histograms represent cells stained with isotype-matched control mAbs.

CHARACTERIZATION OF PML ANTIGEN DELOCALIZATION IN ACUTE PROMYELOCYTIC LEUKAEMIA BY IMMUNOCYTOCHEMISTRY

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Introduction. Most of acute promyelocytic leukaemias (APL) show a rearrangement of PML and RAR α genes resulting in a PML-RAR α fusion gene. Characterization of APL in urgent situation is necessary to get a long term 80% remission in ATRA treatment. Moreover, neither APL is coming from other rearrangement nor APL is not expressing the fusion gene respond to ATRA and the therapeutic profile is completely different. **Objectives.** Fast characterization of PML-RAR α fusion in APL suspicion by immunocytochemical technique. **Material and Methods.** Eighteen patients with acute leukaemia have been tested immediately [M2 AML (n=4), M3 or M3v AML (n=6), M4 AML (n=1)] and lately [NK AML (n=1), M3 or M3v AML (n=6)] by immunocytochemistry by reacting PML antigen with MoAb PG-M3 antibody (LSAB + Alkaline Phosphatase Dako, Denmark). **Results.** Among the immediately analyzed patients, the 6 M3 AML have shown PML protein delocalization while the others have shown a reactivity with the tested antibody without any delocalization evidence. For the lately treated other 7 cases, none of the 3 patients with expression of the fusion gene shows any immunocytochemical reaction with the MoAb PG-M3 antibody. Moreover, 3 patients have NK AML, M3v AML t(8,21), M3v AML t(15,17) show PML antigen delocalization. **Conclusions.** The characterization of PML antigen delocalization is easy to perform, fast, and well suited for routine APL urgent diagnosis. However, later antigen detection is not valid in our series of cases. A complementary study would be necessary in order to determine the analysis delay for PML antigen by immunocytochemistry.

QUANTITATIVE EXPRESSION OF THE THREE EPITOPES OF CD34 IN 300 CASES OF ACUTE MYELOID LEUKEMIA

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The various epitopes of the CD34 molecule have been classified according to their differential sensitivity to enzymatic cleavage by neuraminidase, chymopapain and a glycoprotease from *Pasteurella haemolytica*. Although monoclonal antibodies have been developed that specifically identify these epitopes, few studies have evaluated their distribution and their quantitative expression on leukemic blasts. We report here a prospective multicenter study in which we examined and quantified the expression of the 3 classes of CD34 on fresh leukemic blast cells in 300 cases of AML. The binding of monoclonal antibodies was studied by flow cytometry, allowing appreciation of blast cell positivity as well as their mean fluorescence intensi-

ty. These quantitative data were made comparable between centers by means of a calibration curve established with the same reagents in all laboratories. Quantitative expression of class I molecules was significantly higher than that of class II and class III ($p < 0.0001$). The three classes were more frequently expressed in M0 and M1 and less in M3 and M5. The highest levels of CD34 expression were observed in M2, M0 and M1 and the lowest in M3, M5 and biphenotypic AL for class II and III. CD34 expression was lower for all classes in cases with a normal karyotype, compared to cases with structural or numerical abnormalities. In cases with a t(9;22) the expression of class I was significantly higher than that of class II and III and the opposite was observed in AML with t(15;17). Class III CD34 high intensity appeared to be a marker of good prognosis.

CLINICAL ROLE OF BLAST CELL IMMUNOPHENOTYPE IN ACUTE NON-LYMPHOBLASTIC

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The purpose of this study was to analyze the relationships between the expression of surface markers and clinical characteristics and outcome in patients with *de novo* acute non-lymphoblastic leukemia (ANLL). **Material and Methods.** Blast cells of 120 adult patients with ANLL (14-67 years, Me - 40) were investigated by indirect immunofluorescence using twenty monoclonal antibodies, mainly during the period of 1985-1996. **Results.** Expression of CD13 was associated with a higher mean WBC count (58.8 ± 3.07 vs $23.5 \pm 6.29 \times 10^9/L$; $p = 0.048$). Erythroid antigens HAE-3 and HAE-9 expression correlated with lower hemoglobin level; (6.9 ± 0.47 vs 8.1 ± 0.23 g/dL; $p = 0.039$ and lower platelet count (64.5 ± 14.05 vs $101.2 \pm 10.8 \times 10^9/L$; $p = 0.045$). The younger age of patients was associated with CD33 positivity (37.3 ± 3.69 vs 46.5 ± 2.43 years; $p = 0.037$). All patients were treated with a combination of ARA-C and anthracyclines. Total CR rate was 57.5% and the median overall survival was 6 months; the median CR duration, 10 months. Immunologic markers did not influence the CR rate ($p > 0.05$), however, the highest CR rate was associated with CD10 positivity (89% vs 54%; $p = 0.07$) and CD13 negativity (74% vs 42%; $p = 0.07$). Longer overall survival correlated with CD19 (Me 21 vs 6 months; $p = 0.049$) and CD50 (Me 18.5 vs 0.5 months; $p = 0.0027$) expression. Expression of CD34 and CD7 was associated with shorter CR duration (Me 10 vs 17 months; $p = 0.01$, and Me 5.5 vs 15 months; $p = 0.01$, respectively). **Conclusion.** Immunophenotype of ANLL blast cells can have some influence on the outcome of the disease and perhaps should be taken into account when treating patients with ANLL.

CD56+ EXPRESSION OF ACUTE MYELOID LEUKEMIA MAY PREDICT FATAL OUTCOME IN CHILDREN WITH DOWN'S SYNDROME

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Children with Down's syndrome experience better outcome of acute myeloid leukemia (AML) than other children. We report on a 22-month old girl with Down's syndrome who developed

a CD7⁺CD56⁺ positive AML with an unusually aggressive clinical course. The girl has been previously diagnosed with refractory anemia with excess blasts in transformation (RAEBt). AML was treated using the standard protocol AML-BFM 98, achieving complete remission on day 15 after induction chemotherapy. First relapse was apparent at the end of the first consolidation chemotherapy, with 5% of blasts in the bone marrow biopsy despite bone marrow hypoplasia, and subsequently increasing blast infiltration of the bone marrow. Cytogenetic study showed blasts with 60-61 chromosomes with a complex karyotype in 90% of metaphases analyzed. Fluorescence *in situ* hybridization studies were negative for ABL-BCR. At the age of 26 months she received a bone marrow transplant from a matched sibling donor, without achieving complete remission. Blasts expressed CD45⁺, CD34⁺, CD13⁺, CD7⁺ and were CD33⁻. CD56⁺ was expressed aberrantly. Marrow aspirates disclosed 20% of medium sized blasts with round nuclei, and cytoplasm without granulation. No Auer rods were present. The bone marrow showed myeloperoxidase staining negative blast cells. The girl died at the age of 2 years and 11 months, 7 months after first diagnosis, on day +36 post-transplantation. CD56⁺ is considered a marker of natural killer (NK) cells also expressed in some cases of AML, and may be involved in cell adhesion mediating extramedullary leukemic infiltration. The immunophenotypic similarities of myeloid and NK cell neoplasias suggest a biological relationship between the behavior of both disorders. Extrapolating results from the adult population, AML co-expressing NK cell CD56⁺ antigen may define a prognostically different entity in children with Down's syndrome. Therefore, CD56⁺ expression should be specifically searched for in AML, and may indicate the need for a more aggressive therapeutic approach, even in Down's syndrome children.

CHARACTERIZATION OF AN ACUTE LEUKEMIA DERIVED FROM DENDRITIC LYMPHOID CELLS WITH CD 33 EXPRESSION

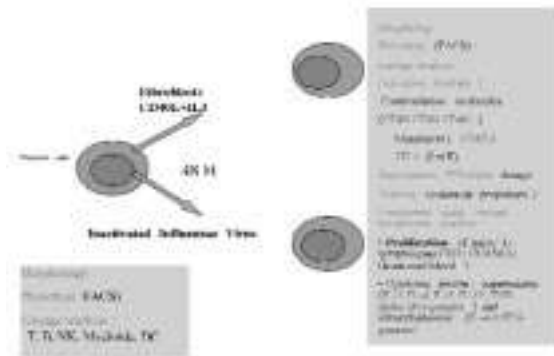
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Introduction. Dendritic cells (DC) are antigen-presenting cells which play a major role in the initiation of immune responses and in the induction of tolerance. Two important DC families have now been described: a DC1 population derived from a myeloid progenitor (CD11c⁺ CD116⁺ CD45RO⁺My⁺) and a DC2 type derived from a lymphoid progenitor (CD11c⁻ CD123⁺ CD45RA⁺ My⁻). The differentiation pathways of DC2 and DC1 have been well documented. Recently a new entity of acute leukemia has been reported: it co-expresses CD4⁺ and CD56⁺ markers without other lineage specific markers (e.g.: myeloid, B and T lymphoid, NK). This and others results implied that the dendritic origin of these leukemic cells was lymphoid (pDC).¹
Aim. We studied leukemic cells from a patient presenting with an acute leukemia with similar morphologic, clinical and immunotypic phenotypic features as described above. However, these cells expressed the myeloid CD33 and the NK CD16 markers. Despite these immunologic differences, we demonstrated that these cells were derived from the pDC lineage.
Patient. A 69-year old man presented with several cutaneous lesions, polyadenopathy and a tricytopenya (WBC: $5 \times 10^9/L$, Hemoglobin 117 g/L, platelets $24 \times 10^9/L$). The peripheral blood and bone marrow blasts expressed CD4⁺, CD56⁺, and CD33⁺.

Control. A 3-year old child presented with an acute myeloid leukemia (monoblastic M5a according to the FAB classification) with typical morphologic and cytochemical features, and CD4⁺ CD56⁺ CD33⁺ expression. **Materials and Methods** (see the Figure below). **Results.** The morphologic features of blasts cells were similar to those previously published; after overnight culture with IL3 in the presence of a fibroblastic feeder layer transfected with a CD40 ligand construct, we obtained dendritic differentiation; the resulting immunophenotype was: CD123⁺ BDCA2⁺ BDCA4⁺ CD45RA⁺ CD45RO⁻ HLA-DR⁺; we therefore observed differentiation to mature DC with increasing expression of co-stimulation markers (CD40, CD80, CD86), HLA-DR and HLA-Class I; the functional ability to activate the naive T lymphocytes (purified from cord blood) leading to a lymphoid response with a Th2 polarization (IL5 production, detection of intracytoplasmic IL4) was shown. Control cells did not differentiate. **Discussion.** We suggest that only leukemic cells from the patient (and not from the control) were able to differentiate into the lymphoid dendritic lineage. CD33 expression was positive with the MAb LeuM8 (Becton Dickinson, PE, USA), and negative with the MAb WMS4 (Dako, FITC). CD33 expression appeared over time in culture. Standardization of the MAb method to detect CD33 expression with a sufficiently high sensitivity is required. **Conclusion.** We suggest that CD33 expression should not be an exclusion criterion for the new leukemic entity CD4⁺ CD56⁺. This group could be named as a lymphoid dendritic acute leukemia (LDAL).

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IMMUNOPHENOTYPE OF MYELOBLASTIC LEUKEMIAS IN THE GOELAMS PROTOCOLS. A PROSPECTIVE STUDY ON 450 CASES

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There is still debate in the international community about the panel of monoclonal antibodies necessary to perform proper lineage assignment of leukemic cells and identify immunophenotypes related to prognostic factors and/or useful for

the follow-up of chemiosensitivity and of minimal residual disease. Extensive immunophenotyping was performed in a multi-center study by the Groupe d'Etude Immunologique des Leucémies (GEIL) in two prospective therapeutic protocols of the French collaborative group GOELAMS. This allowed us to examine the distribution of immunophenotypic features of 450 acute myeloblastic leukemias. Lineage assignment can be achieved by using the four markers CD13 (membrane and/or cytoplasmic), CD33, CD117 and myeloperoxidase. Although in most cases at least two of these markers are expressed, evidence was obtained of rare patients with cells expressing only one of these four antigens. The use of monoclonal antibodies to more mature differentiation antigens afforded clinicians more detailed information about the lineage engagement of the cells (monocytic, granulocytic, dendritic). Lymphoid-lineage associated markers allowed identification of aberrant co-expression and a few cases of biphenotypic acute leukemia. Being achieved in a homogeneous therapeutic protocol, these immunophenotypic studies will prove of interest in relation to patients' evolution.

MORPHOLOGIC AND IMMUNOLOGIC CHARACTERISTICS OF BIPHENOTYPIC ACUTE LEUKEMIA: A MULTICENTER STUDY OF 57 CASES

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Among 2,302 consecutive acute leukemias, 57 cases were validated as biphenotypic acute leukemia (BAL) according to modified EGIL criteria, including 55 *de novo* and 2 therapy-related cases. Two subtypes were defined *BAL1* (29 cases) associated an undifferentiated morphology, negative myeloperoxidase reaction (MPO), lymphoid (Ly) and myeloid (My) markers with B or T-cell Ly score >2 and at least 1 major B- or T-cell markers (i.e. CD22, cCD79a, cμ, cCD3) and My score >2 (or My score =2 and CD36+). *BAL2* (28 cases) were characterized by a My morphology (Auer rods, MPO+ or monocytic esterases) and Ly markers similar to *BAL1*. The overall incidence of BAL was similar in children (15/549; 1.8%) and adults (42/1753; 2.2%). In comparison with 61 AML-M0, *BAL1* exhibited a similar incidence of granulocytic dysplasia (21%) and My marker expression except more CD65+ (41% vs 16%) but less CD117+ (30% vs 66%). *BAL2* showed more FAB-M1 type (71% vs 30%), fewer Auer rods (11% vs 34%) and more CD7+ (39% vs 16%) and CD34+ (89% vs 54%) but similar myelodysplastic features and My marker expression as 480 non-biphenotypic AML. According to B- or T-cell Ly markers we recognized 43 *BAL/B* and 14 *BAL/T*. Ly marker expression was similar in *BAL1* and *BAL2*. In comparison with non-biphenotypic ALL, the incidence of CD10+ was lower and CD34+ higher in BAL. In addition, *BAL/B* were more frequently classified as B1/pro-B EGIL subclass than 284 B-ALL (35% vs 8%). As well, immature EGIL T1/TII subclasses were more frequent in *BAL/T* than in 51 T-ALL (79% vs 16%). PCR analysis revealed a complete or partial (DJ) immunoglobulin heavy chain gene rearrangement in 10 out of 13 *BAL/B*.

However no rearrangements of either TCR γ or δ genes were observed in a *BAL2/TII*. Follow-up data could be obtained in 15 children, 23 adults from 18-60 years old and 17 over 60 years old. The CR rate was 100%, 74% and 29%, respectively. The EFS at 2 years was 40%, 32% and 0% for a median follow-up of 31, 13 and 4 months (33%, 30% and 0% being allografted). In conclusion, BAL is a rare subset of AL with immaturity of both My and Ly components associated with a worse prognosis, even in children.

INTEREST OF IMMUNOPHENOTYPING IN ACUTE MYELOID LEUKEMIA

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Background. The acute leukemias are a heterogeneous group of neoplasms affecting uncommitted or partially committed hematopoietic stem cells. The retained capacity of some differentiation is the basis for the phenotypic classification. Flow cytometry is a widely used method to diagnose and classify acute leukemias, thereby complementing morphology and cytochemistry. **Material and methods.** We immunophenotyped 170 cases of AML from September 1996 to April 2001. Only 45 AML patients (without promyelocytic leukemia-APL) were included in this study. For each patient we analyzed several characteristics such as: FAB morphology, WBC count at diagnosis, platelet level, Hb level, karyotype and immunophenotype. Blast cells from 45 cases were analyzed with a uniform panel of monoclonal antibodies directed at myeloid, lymphoid, stem cell and other leukocyte antigens with a Facscalibur (Becton Dickinson). **Results.** These patients showed a full expression of panmyeloid phenotype, defined by the expression of all 3 immature myeloid markers: CD13 -98%, CD33 -98% and CD117 -82%. Other markers, such as CD65-67%, CD14-24% and CD15-47%, were less commonly positive and their expression were rather in AML2 and AML4 subgroups of AML in FAB classification. The expression of CD4-62% was uniform in the AML subgroups. We attempted to evaluate the prognostic significance of 3 MoAbs in particular. We found that CD7 was positive in 36% of AML; CD34 in 60% and CD56 in 27%. **Conclusions.** The diagnostic role of immunophenotyping is unquestionable. Immuno-

Myeloid markers	%	T Lymphoid markers	%	B Lymphoid markers	%	NK Lymphoid markers	%
MPO	86	CD2	2	CD19	11	CD56	27
CD13	98	CD3 surface	0	CD20	1	CD16	9
CD14	24	CD3 intracyto	0	CD22 surface	0	-	
CD15	47	CD4	62	CD22 intracyto	0	other markers	
CD16	9	CD5	0	anti P glyco.	0	CD10	0
CD33	98	CD7	36	CD79a	14	CD34	60
CD36	57	CD8	0			HLADR	
89							
CD65	67						
CD117	82						
Gly. A	4						

Gly. A=glycophorine A; anti P glyco.=anti P glycoproteine.

phenotyping combined with morphology and cytochemistry has become a critical tool and is essential for characterizing and subclassifying AML appropriately. Flow cytometry completes the morphology and highlights several blast cell populations by CD45 gating. Immunophenotyping studies have been decisive in characterizing and classifying a small subgroup of poorly differentiated acute leukemias. It is also useful in characterizing and classifying AML with monoblastic/monocytic differentiation. (CD14 and CD36 can be useful markers in characterizing AML with monocytic component). The contribution of immunophenotyping to prognosis in AML has been a controversial issue. Nevertheless, it seems that the role of immunophenotyping in predicting the response to treatment in AML is indirectly important through its contribution in characterizing particular subtypes with proven or presumed bad prognosis. It is still important to practice and study systematically immunophenotyping, particularly for CD34, CD7 and CD56.

SESSION E LYMPHOPROLIFERATIVE DISORDERS

CHRONIC LYMPHOPROLIFERATIVE DISORDERS

Matutes E

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The chronic lymphoproliferative disorders comprise an heterogeneous group of diseases that arise from the clonal expansion of lymphoid cells committed to the B or T lymphoid lineages. They can be classified into two main groups: primary leukemias and leukemia/lymphoma syndromes or the leukemic phase of non-Hodgkin's lymphomas. The precise diagnosis of the various disease entities should be based on a multiapproach analysis that integrates clinical and laboratory features. The latter should include: (1) cell morphology in fresh and well spread peripheral blood made films; (2) immunologic markers; (3) histology of the tissues involved; (4) molecular genetics and, when relevant, virological studies for EBV or HTLV-I. Immunophenotyping by applying a battery of monoclonal antibodies (McAb) that identify a variety of membrane and cytoplasmic antigens in the lymphocytes is a key test for disease classification and characterization and should be carried out in the routine diagnosis. Markers allow, on the one hand, to establish the B or T cell nature of the neoplastic cells and distinguish mature (TdT⁻) from immature/blastic (TdT⁺) lymphoid malignancies and, on the other hand, disclose immunophenotypic profiles which are characteristic of certain disorders. This is the case of chronic lymphocytic leukemia (CLL) where neoplastic cells have a characteristic immunologic profile, e.g. weak Smlg, CD5⁺, CD23⁺, FMC7⁺ and weak or negative expression of CD22 and CD79b, which when compounded in a scoring system allows CLL to be distinguished from other B-cell conditions. Similarly, the immunophenotypic profile of hairy cell leukemia (HCL) is different from that of splenic lymphoma with villous lymphocytes and the variant form of HCL when the reactivity of four markers: CD11c, CD25, CD103 and HC2 is considered. Other McAbs of diagnostic relevance are those that detect cyclin D1 in the nucleus of the neoplastic cells. The expression of cyclin D1 can be estimated by immunocytochemistry or flow cytometry. Both techniques have pitfalls but overall are reliable for the detection of this cyclin. Analysis of cyclin D1 is important as most cases of mantle-cell lymphoma will be positive whilst most of the other B-cell diseases are cyclin D1 negative. Still, results need to be compounded with other laboratory tests, eg FISH assessing t(11;14) or histology to make a definitive diagnosis of mantle-cell lymphoma as cyclin D1 may be expressed, albeit weakly, in other diseases with or without t(11;14) such as prolymphocytic leukemia, hairy cell leukemia and a few cases of splenic lymphoma with villous lymphocytes. In T-cell malignancies, there is more overlapping in marker expression. Nevertheless, some markers are more consistently and strongly expressed in certain conditions. For instance strong CD7 reactivity and co-expression of CD4 and CD8 in T-PLL, expression of CD8 and CD57 in large granular lymphocyte leukemia (T-cell-LGL), a natural killer (NK) immunophenotype in NK-LGL, or strong CD25 expression in HTLV-I⁺ T-cell leukemia/lymphoma. NK cell leukemias and lymphomas encompass a variety of disease entities that include cases of NK-LGL, a subset of nasal and angiocentric lymphomas

and perhaps and more rarely some acute leukemias. In addition to the diagnostic relevance of the immunophenotyping, some markers have been shown to have a significant prognostic importance. Examples of these are the following: CD38 expression in neoplastic CLL lymphocytes which has been documented to be a prognostic factor for survival, independent of other variables including IgHV mutations; it is important that CD38 is estimated in the malignant CLL lymphocytes by a triple platform flow cytometry method with the McAb CD19, CD5 and CD38; p53 protein expression which in most cases of low and high grade malignancies often correlates with mutations/deletions of the tumor suppressor gene that codes for the protein, disease progression and/or resistance to therapy; Ki-67, a marker which measures the proliferative rate of the tumor. Finally, studies on the expression of certain antigens, such as CD79b or the family of bcl-2 proteins may give insights into the pathogenesis of some disorders and others, eg CD52 or CD20 must be tested when a patient is considered a candidate for antibody therapy. We will describe the features of the various lymphoid disorders and emphasize the value of the immunologic markers for the precise diagnosis of these conditions.

B-CELL CHRONIC LYMPHOPROLIFERATIVE DISORDERS: THE GEIL EXPERIENCE

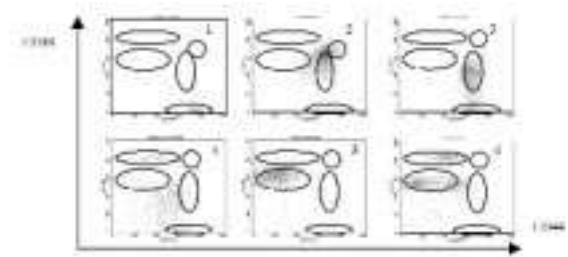
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The classification of B-cell chronic lymphoproliferative disorders has great clinical and epidemiological importance. Moreover, because prognosis is variable from one nosological entity to the other, the differential diagnosis has important therapeutic implication. For this purpose, morphology plays a key role. Nevertheless, over more than thirty years, several morphologic classifications have been proposed, reflecting the difficulties, in part due to the subjectivity of the observers. Genetic techniques (karyotype, fluorescence *in situ* hybridization, molecular biology) can be very helpful in this differential diagnosis. Immunophenotyping is beginning to have an important role, especially in *atypical* cases. Interpreting the immunophenotype of chronic B-cell lymphoproliferative disorders has highlighted the need for data on B-cell subpopulation ranges from healthy subjects, not only in terms of percentages of positive cells, but also in terms of expression intensity. Indeed, flow cytometric (semi-) quantification of cell surface markers appears to be a very important parameter in the diagnosis of chronic B-cell disorders. Clarification of methodological procedures is necessary to reach reproducible results in multicenter studies, especially in terms of fluorescence intensity interpretation. A CLL scoring system using a few markers (CD5, CD23, FMC7, surface κ/λ , CD22 and/or CD79b) is very helpful for the differential diagnosis between *typical* CLL and the other B-cell chronic leukemias and NHL. In the GEIL multicenter experience, CD22 and CD79b have the same diagnostic power. Among the other CD5 positive B-cell chronic lymphoproliferative disorders, atypical CLL and mantle cell lymphoma immunophenotypes are very close, suggesting, in conjunction with genetic data, a possible common pathway for these two diseases. Only CD54 seems to be more intensely expressed on MCL cells than on atypical CLL ones. Follicular lymphoma cells have an abnormal very specific immunophenotype (low expression of CD44 and CD38 as shown in histograms 4, 5 and 6; the

three first profiles are observed in other B-cell chronic lymphoproliferative disorders). This combination can be used for minimal disease contaminated samples. In conclusion; 1. flow cytometry improves the characterization and the immunologic diagnosis of B-cell malignant lymphoproliferative disorders; 2. flow cytometry allows the detection of minimal contamination and residual disease follow-up; 3. immunophenotype could provide prognostic and physiopathologic data. This needs to be further studied on more samples.



VALUE OF ADHESION MOLECULE AND BCL-2 EXPRESSION FOR THE DISTINCTION BETWEEN BURKITT'S AND NON-BURKITT'S (OR BURKITT-LIKE) LYMPHOMAS

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The differential diagnosis between Burkitt's lymphoma (BL), especially atypical forms, and some medium-sized cell lymphomas (BL-like or BLL) displaying features of BL, such as a high proliferation index, cohesiveness, monomorphism, basophilic cytoplasm, rearrangement of c-myc, remains unresolved on morphologic grounds only. The recent WHO classification does not enlighten this point. However the diagnosis has therapeutic and prognostic implications: BL can be cured by specific chemotherapy whereas BLL bears a worse prognosis, and an urgent response of the laboratory is usually required in order to begin the appropriate treatment rapidly. BL have been reported not to express adhesion molecules and BCL-2 is consistently negative. With the aim of helping the discrimination between BL and BLL, we studied the expression of CD11a/LFA-1 α , CD18/LFA-1 β , CD44 homing receptor, CD54/intercellular adhesion molecule, CD62L/ECAM-1, BCL-2 and p53 proteins, by cytometry and immunocytochemistry, in addition to the classical panel including CD10, CD38, KI-67. From our files, we selected 15 BL/BLL cases of adult patients (16-77 yrs) with cytological, immunologic and cytogenetic study (12 with available histology). Tumor cells were studied in lymph nodes (6 cases), fluids (5 cases), extranodal sites (3 cases), and bone marrow (1 cases). Among them, 8 were classified as BL (typical or atypical), 7 as BLL, 2 could not be classified by cytology alone. In BL, CD38 was always positive, BCL-2, CD11a, CD18, CD44, CD54, CD62L, p53 always negative. In BLL, CD38 was negative in 2/7 cases, BCL-2 was positive in 6/7 cases, CD11a positive in 5/7, CD18 was positive in 5/7, CD44 positive in 4/7, whereas CD54 and CD62L were negative in all cases. In the 2

doubtful cases, phenotype points for BL in 1 case (BCL-2 and adhesion molecules negative) and for BLL in the other (BCL-2 negative, but CD11a/CD18/CD44 positive). Cytogenetic analysis demonstrated translocation involving 8q24 in all cases, associated with rearrangements of 3q27 and/or 18 q21 and/or 1q21 in 6/7 BLL cases. Six of the 8 cases of BL are alive in CR, whereas 6/7 BLL are dead. Even if these results need to be confirmed on a larger series of patients, we think that the search for BCL-2 and adhesion molecule expression constitutes a helpful tool which can aid diagnosis in this difficult field.

RELATION OF CD38 EXPRESSION WITH DIAGNOSIS OF DE NOVO CHRONIC LYMPHOCYTIC LEUKEMIA

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Introduction. Chronic lymphocytic leukemias (CLLs) are heterogeneous diseases. Few of them present clinically with aggressive features. Many of them have an indolent evolution without a significative diminution of life expectancy. In 1999, Damle *et al.*¹ described a correlation between CD38⁺ expression, the IgH gene germinal status (mutated or unmutated) and a more aggressive evolution. Although the relationship between CD38 expression and IgH mutation status has been questioned by some authors, the relationship between CD 38 expression and prognosis status seems to be generally accepted as an independent prognostic marker. We performed a study of CD38 expression in a series of *de novo* CLL patients diagnosed in Besançon during a period of 18 months (2000 February - 2001 October). We present a statistical analysis of CD38 status, clinical features (Binet staging), and other biological parameters at time of diagnosis. *Patients.* Seventy-one CLL patients were included in the Besançon study. *Materials and methods.* Immunologic studies were performed using cytometric meth-

Results of the different clinical and biological parameters versus CD38 status in CLLs.

Studied parameters	CD38 - Group (75% of studied CLLs)	CD38+ Group (25% of studied CLLs)
Age (years)	66.5	62.5
Sex ratio M/F	1/0.9	1/0.7
Lymphocytes (x10 ⁹ /L)	20046	20127
Hemoglobin (g/dL)	13.5	13.5
Platelets (x10 ⁹ /L)	208	187
LDH (UI/L)	402	418
β2 microglobulin (mg/l)	4	4.5
Matutes scoring		
4 and 5	28 %	35 %
6	72 %	65 %
Binet staging		
A	80.6 %	72 %
B and C	9.4 %	28 %

ods (FacsCalibur-Becton Dickinson). Fresh peripheral blood was marked using triple or quadruple immunoconjugating colors. Diagnoses with Matutes scoring ≥ 4 were included. CD38 (Ieu17, Becton Dickinson, PE) was considered positive if it was expressed by more than 30% of CD19⁺ cells. CD38 expression was found in 25% of CLLs when applying a cut-off of 30% positivity and in more than 40% if the cut off was 20%. *Conclusion.* CD38 expression is highly variable depending on the cut-off applied on the CD19⁺ cells. This result thus requires standardization of the cut-off (20%? 30%?). The biologic parameters (blood counts, morphologic features, Matutes scoring) were not statistically significantly correlated with the CD38 status. There were no correlations between CLLs presenting atypical/ mixed features, plasmacytoid aspects or CD38 status (results not shown). On the other hand, CD38 expression and the clinical Binet scoring showed a strong correlation. This was dependent upon disease stage (B and C groups being more frequent in the CD38⁺ group).

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SIMULTANEOUS OCCURRENCE OF MYCOSIS FUNGOIDES/SEZARY SYNDROME AND CHRONIC LYMPHOCYTIC LEUKEMIA

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A 74-year old man was referred to our institution for evaluation of multiple itchy and reddish plaques. Cutaneous biopsies revealed lymphoid infiltration consistent with mycosis fungoides. Peripheral lymphocytosis was observed, and flow cytometric immunophenotyping was performed. This, surprisingly, showed a B-cell lymphoproliferative process, CD5 positive, CD23⁺, score 5 in Matutes scoring system, consistent with chronic lymphocytic leukemia. Flow cytometric analysis revealed simultaneous occurrence in the peripheral blood of CD4⁺ cell, with decreased expression of CD3. T-cell receptor molecular analysis showed the same clonal rearrangement in skin and in both the skin and in peripheral blood, confirming the Sézary syndrome. A few months later, surgical removal of an adenopathy was decided, and molecular analysis confirmed the co-existence of B and T clonal rearrangement. Simultaneous occurrence of T-cell lymphoma as mycosis fungoides with an other B pathology has already been described. In this case flow cytometry allowed clear definition of the B and T proliferation. Numerous possible explanations for the association of B- and T-cell lymphomas can be put forward, such as genetic predisposition, viral infection (HTLV-1, EBV), chemotherapy-induced carcinogenesis, malignant T helper cells stimulating a B-cell clone or alterations in progenitor cells before B- and T-cell lineage determination.

CONTRIBUTION OF FLOW CYTOMETRY IN THE DIAGNOSIS OF HODGKIN'S LYMPHOMA

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Introduction: Hodgkin's disease is the most characterized malignant lymphoma. Until now, its diagnosis requires histopathologic techniques and is based on the recovery of elementary lesion (Reed-Sternberg cell CD15, CD25, CD30) dispersed on a reacting context. **Objectives.** To evaluate usefulness of flow cytometry in Hodgkin's disease diagnosis. **Material and methods.** 30 lymph nodes (24 scleronodular, 4 mixt cellularity, 2 nodular lymphocytic predominance) have been tested in comparison with 6 reactive lymph nodes. The lymph nodes have been grinded. Then, the resulting cell suspensions have been washed and incubated with a combination of 8 antibodies before flow cytometry analysis (FacsCalibur, Becton Dickinson). **Results.** The 30 lymph nodes were positive for CD30. A CD4/CD8 mean ratio has been calculated for the two evaluated populations (see table below).

	Reactive lymph node	HD with nodular lymphocytic predominance	Scleronodular HD	Mixed cellularity HD
n	6	2	24	4
Mean ratio CD4/CD8	3.2	6.8	6.9	1.5

Conclusions. Mean ratio of CD4/CD8 > 3.2 among suspected cases of Hodgkin's disease allows presumptive diagnosis of scleronodular and nodular lymphocytic predominance Hodgkin's disease. Consecutively, flow cytometry appears to be a helpful technique to confirm a difficult diagnosis.

CLINICAL AND BIOLOGICAL CHARACTERISTICS OF TWO CASES OF B-CELL CHRONIC LYMPHOCYTIC LEUKEMIA WITH CD8 EXPRESSION

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B-cell chronic lymphocytic leukemia (CLL) with CD8 expression is a rare subgroup described in the literature as a stable disease. We report two patients (males, ages 64 and 70) of our 167 B-CLL cases (1.19 %) who co-expressed CD8 on B cells (CD19⁺, CD20⁺, CD5⁺, CD23⁺, slg dim- κ light chain) with lower density than normal T-cells. They were diagnosed with B-CLL according to N.C.I. criteria. The first case (BP) presented tumoral splenomegaly, hepatomegaly at 2 cm under costal edge, cervical, supraclavicular and abdominal lymphadenopathies, Hb=12 g/L, white blood cells 84.5×10⁹/L with 95% lymphocytes, platelets 26×10⁹/L - stage C/IV (Binet/Rai). The B cells (95% of lymphocytes) were also CD22⁺ (moderate), CD38⁻, CD11c⁺. He received monthly chemotherapy cycles with cyclophosphamide, vincristine and prednisone and every two or three months afterwards. He was well 47 months after his diagnosis, with normal blood cells count, no organomegalies or

lymphadenopathies. Another case (C.T.) was admitted to the Fundeni Clinical Institute with bone tumors (right shoulder and femur). Chest and abdominal computed tomography revealed tumoral lymphadenopathies. A complete blood count revealed normal hemoglobin and platelets, white blood cells 34.7×10⁹/L with 75% lymphocytes. The stage at diagnosis was A/II (Binet/Rai staging systems). The B cells (89% of lymphocytes) were also CD22⁻, CD38⁺, CD11c⁻, CD79b⁺, FMC-7⁻. He received 3 chemotherapy cycles with cyclophosphamide, vincristine and prednisone with a decrease in malignant lymphocytes, regional radiotherapy with 75% decrease of shoulder tumor, followed by 2 cycles with fludarabine. Six months after diagnosis he was admitted to hospital with acute pneumonia. Afterwards he had bone pain and presented evidence of new bone lesions (cervical and lumbar vertebrae, ribs and humerus) with spontaneous fracture of the left humerus. His blood cell count was normal. He died 13 months after diagnosis with progressive bone disease. Unlike most cases reported in literature, our cases had abdominal lymphadenopathy abdomen. Both patients had indolent hematologic disease, but the second patient had bone disease since diagnosis.

USEFULNESS OF FLOW CYTOMETRY ANALYSIS IN ADDITION TO MORPHOLOGIC ANALYSIS FOR B-CELL NON HODGKIN'S LYMPHOMA

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Recently an international consensus on flow cytometry analysis (FCA) was reached to evaluate hematology neoplasms. Although FCA is becoming widely used to characterize lymphomatous proliferations, its use in the diagnosis of non-Hodgkin's lymphoma (NHL) tissue infiltration is still matter of debate. In order to test the usefulness of FCA to obtain a diagnosis we used FCA to study 84 biopsies from patients suspected of having a lymphoma. Cell suspensions were obtained from the biopsies, triple immunolabeling was performed with the CD19 marker in combination with 2 other markers allowing us to analyze the expression of CD5, CD10, CD11c, CD20, CD22, CD23, CD25, CD38, CD43, CD79b, CD103, FMC7, IgM, IgD, IgG, IgA, κ and λ light chain on B-cells. This panel was completed by a CD3/CD4/CD8, CD3/CD7/CD4 and CD2/(CD16⁺CD56)/CD3 triple immunolabeling to evaluate the T and NK populations as well as to detect a loss of a T-cell marker in T-cell lymphoma. Analyses were performed on a COULTER EPICS XL-MCL flow cytometer. Five thousand events were acquired in an electronic gate defined on the FS/SS dot plot corresponding to the lymphocytes. The sum of B, T and NK lymphocytes in the gate should be above 80% to allow further analysis. Cytological imprints and tissue processing had been done for all cases. Morphologic diagnosis obtained by cytology, histology and immunohistochemistry was compared to the results of the FCA. Fifty cases were peripheral NHL: 44 B-cell and 6 T-cell ; 28 biopsies displayed an inflammatory process (21 non-specific lymphadenitis, 2 sarcoidosis, 1 Whipple's disease, 1 Castleman's disease, 2 tuberculosis, 1 Epstein-Barr virus associated atypical lymphoid hyperplasia). In B-cell NHL 40 cases out of 44 were concordant between FCA and morphologic diagnosis. The 4 discrepant cases were difficult cases: 2 with an abundant normal T-cell component, 1 was a marginal zone lymphoma which had developed after immunosuppressive treatment and one was an heterogeneous infiltration of MALT lymphoma in

the thyroid. Of the 6 cases of T-cell NHL, 2 cases failed to be diagnosed by FCA. There were no false positive cases of malignancy detected by FCA. From this study of 84 lymphoid tissue biopsies we conclude that FCA can be used at first level to detect B-cell lymphoid neoplasm at diagnosis in combination with cytology since the histology allowed classification of the NHL and systematically confirmed the diagnosis obtained by FCA. Such a strategy would be useful to have a rapid and accurate diagnosis.

ANTIGEN PRESENTATION PATHWAY VIA HLA CLASS II MOLECULES IS MODIFIED IN CERTAIN NON-HODGKIN'S B LYMPHOMAS

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Non-Hodgkin's B lymphomas (B-NHL), in contrast to most solid tumors, express HLA class II molecules. This expression may allow tumor B-lymphocytes to interact with, and present tumoral antigens to CD4⁺ T-lymphocytes. In this context, in order to identify possible anomalies in the antigen presentation pathway, we characterized the expression of HLA class II molecules in B-NHL compared to in normal B splenocytes. Specifically, we studied by flow cytometry and Western blot, the different populations of HLA class II molecules expressed on tumor B-lymphocytes of 16 patients. We also examined the localization of HLA-DR molecules in different intracellular compartments by confocal microscopy. We show evidence for two different groups of B-NHL based on HLA class II expression profile compared to that of normal B-lymphocytes. A first group comprised follicular and lymphocytic B lymphomas, which presented no major differences from normal B-lymphocytes (10 patients). A second group, comprised of mantle and diffuse large cell B-NHL (6 patients), showed marked differences from normal B-lymphocytes: HLA-DR/CLIP complexes were not present at the cell surface, HLA-DR molecules were localized in lysosomes and a reduced level of total HLA-DM and DO was observed. These results suggest, for the second group, a modification of the antigen presentation pathway that may have a functional impact on the capacity of these B-NHL cells to interact with CD4⁺ T-lymphocytes.

BENIGN MONOCLONAL T LYMPHOCYTES

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Introduction: In the study of lymphocytosis or neutropenia by flow cytometric immunophenotyping (FCI) we can detect some abnormalities of T-cells, which can reflect a monoclonal disorder. The aim of the present study was to evaluate the monoclonal character of some aberrant T-cell immunophenotypes by molecular biology of the T-cell receptor (TCR) and to assess its clinical significance. **Material and methods:** Peripheral blood of 8 patients with lymphocytosis or neutropenia was submitted for FCI. The samples were analyzed for various antigens (CD2, CD3, CD5, CD7, CD45, CD4, CD8, CD19, CD56, CD16, CD57, αβ, γδ), using a FacScan flow cytometer. These patients were all asymptomatic and had normal hemoglobin and platelet counts. **Results:** FCI identified a variable percentage (10 to 70% of total lymphocytes) of T-cell subsets with some abnormalities. All expressed CD2. CD3 was positive in 6 patients and negative in the other 2. Four patients presented CD4⁺/CD8⁺, two patients CD4⁺/CD8⁻, one CD4⁻/CD8⁻, and the other one CD4⁺/CD8⁺. None had CD16, 6 cases expressed CD57 and CD56 in 3 cases. The expression of CD5 and CD7 was of variable intensity. Molecular biology of the γ chain of TCR demonstrated a clonal band by the polymerase chain reaction technique in all the cases. Seven, 8, 14, 16, 17, 25, 26 months and 8 years have passed since the diagnosis, and all the patients remain asymptomatic. **Conclusions:** T lymphoproliferative disorders (TLD) have been considered to have an aggressive course and a bad prognosis. Nevertheless, with FCI and molecular biology confirmation, we can detect a group of patients with monoclonal T-cell subsets and a benign course. This would reflect a TLD of unknown significance like monoclonal gammopathy of unknown significance (MGUS) in B-cells. Long-term follow-up of these patients is required to assess the probably benign nature of this entity.

B-CELLS OF LYMPHOPROLIFERATIVE DISORDERS EXPRESS A RECEPTOR FOR THE MITOGENIC HEPARIN AFFINITY REGULATORY PEPTIDE

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Background. Heparin affinity regulatory peptide (HARP) belongs to the family of heparin-binding molecules. It presents mitogenic activity for many cells including fibroblasts, endothelial and epithelial cells. In this study, we looked for its expression by peripheral blood mononuclear cells from normal subjects and from patients suffering from lymphoproliferative disorders. **Patients and Methods.** Peripheral blood mononuclear cells of 10 normal subjects, 10 patients with non-Hodgkin's lymphoma (NHL), and 18 with chronic lymphoid leukemia (CLL) were analyzed after incubation in the absence and in presence of 1 μg/mL of recombinant HARP. After washes in PBS, cells were incubated with a goat anti-HARP antibody followed by a

FITC-conjugated swine anti-goat. Normal goat serum was used instead of primary antibody to serve as a negative control. Multiple immunofluorescence stainings were performed by coupling an anti-CD19-PE and/or an anti-CD2-PC5 on this first staining in order to identify the cell population expressing HARP. After washes in PBS, cells were fixed with 1% formalin and analyzed with an Epics XL flow-cytometer (Beckman, Coulter). *Results.* HARP was not found on the surface membrane of lymphocytes, monocytes, polymorphonuclear cells or platelets. After incubation in 1 $\mu\text{g}/\text{mL}$ of recombinant HARP, HARP

expression was only observed on B-lymphoid cells from normal and pathologic subjects without any difference between the different lymphoproliferative entities. No staining on T-cells, polymorphonuclear cells or platelets was found. *Conclusions:* We show for the first time that B lymphoid cells are able to retain, on their surface, the mitogenic factor HARP which is known to be implicated in angiogenesis and the proliferation of immune cells. These preliminary results need to be confirmed in a larger series of patients and justify the study of cell interactions both *in vivo* and *in vitro* after cell sorting.

Index of authors

name, page

- Achour A 19
 Aho S 12
 Amiot L 4
 Arnoulet C 14
 Aymerich M 2, 5
- Barritault D 19
 Baseggio L 16
 Bellosillo B 2
 Béné MC 1, 3, 8, 12, 14
 Berger F 16
 Bernard M 4
 Bernier M 12
 Billot M 17
 Bissieres P 18
 Boixadera J 19
 Bosque G 19
 Boucheix C 8
 Boudard D 6
 Breitenbach L 18
 Brière F 11
 Brignole-Baudouin F 19
 Brion A 17
 Brunet C 1, 2, 12
 Buisine J 14
 Bulabois B 7, 13, 17
- Cabezudo E 19
 Callet-Bauchu E 16
 Camoin L 1
 Campo E 2, 5
 Campos L 6, 9, 12
 Cantalejo MA 13
 Carli PM 2, 3, 12
 Carreras E 5
 Casasnovas RO 10, 14
 Castillo M 2
 Cela E 13
 Chaouchi N 8
 Chaperot L 13
 Charbonnier A 4
 Chatelain B 3, 9, 12, 18
 Chatelain C 12
 Chautard S 6
 Chehata S 7
 Cheze S 14
 Ciudad J 9
 Clody P 17
 Colita DN 18
 Collonge MA 7
 Colomer D 2, 5, 19
 Conciatori M 1
 Corcos L 7
 Cornet Y 12, 18
 Courty J 19
 Cuquemelle F 1
 Czerkiewicz I 18
- D'Hautcourt JL 1
 Daliphard S 12, 14
- Darodes de Tailly P 7, 13, 17
 Davis BH 10
 de Lamballeri XN 11
 Deconinck E 13
 Deneys V 12, 16
 Dhello G 18
 Díez JL 13
 Dignat-George F 1, 2
 Dina J 14
 Dioucoure D 18
 Drénou B 4
 Dromelet A 9, 18
 Dübel S 3
 Dumitrescu AM 18
- Ennabli S 7
 Entz-Werlé N 6
 Escoda L 19
 Esteve J 5
- Falkenrodt A 6, 12
 Fauchet R 4
 Faure GC 3, 8, 12, 14
 Fekih S 7
 Felman P 16
 Fenneteau O 14
 Fernández-Cruz E 13
 Feuillard J 9, 18
- Gambert P 7, 8
 Garand R 14
 Garban F 19
 Garnache F 7, 13, 14, 17
 Gaugler B 4, 11
 Georgescu O 18
 Gerland L 12
 Gil J 13
 Girodon F 2, 3, 12
 Goffinet C 12, 18
 Grange F 17
 Grosset JM 4
 Guyot M 7
 Guyotat D 6
- Harle JR 1
 Hélias C 6
 Hennaux V 12
 Husson B 9
- Imbert M 14
 Isnardon D 4, 11
- Jacob MC 12
 Jarrossay D 11
 Jenhani F 7
 Jouault H 9, 14
- Kahn E 7
 Karawajew L 5
 Kennel de March A 8
 Ketelslegers O 12
 Kolopp Sarda MN 8

- Kouassi D 3
 Kühlein E 12

 Laatiri MA 7
 Lafage-Pochitaloff M 4, 11
 Lamy T 4
 Latger-Cannard V 14
 Lavabre-Bertrand T 2
 Le Friec G 4
 LeCalvez G 12
 Lemaire-Ewing S 7
 Lepelley P 9, 14
 Leporrier M 14
 Lessard M 6
 Leymarie V 6
 Liénard A 7
 Lizard G 2, 7, 8
 Llorente A 19
 López-Berges MC 9
 López-Guillermo A 5
 Ludwig WD 5
 Lutz P 6

 Macro M 14
 Magniez N 19
 Mallet M 14
 Mannone L 2
 Markina I 12
 Martin A 18
 Martinez A 2
 Matutes E 15
 Mauvieux L 6
 Maynadié M 2, 3, 7, 9, 12, 14
 Mazurier I 17
 Merlin M 4
 Mohty M 4, 11
 Moicean AD 18
 Monier S 7, 8
 Montserrat E 5
 Morel D 16
 Moskovtchenko P 12, 17
 Mounier C 6

 Nacol-Lizard S 8
 Néel D 7
 Niculescu R 18
 Nitu G 18

 Olive D 4, 11
 Orfao A 9
 Ortin X 19
 Oulai U 3

 Pages J 16
 Pagniez M 1
 Pangault C 4
 Parisi E 17
 Pasquier MA 19
 Pédrón B 14
 Pérez I 13
 Philip P 12, 14

 Picard F 9
 Piselli S 6
 Plouvier E 7
 Plumas J 19
 Poncelet P 2
 Potie C 1
 Poulet J 7, 13, 17
 Preudhomme C 14
 Raphael M 18
 Reman O 14
 Renoir C 18
 Rodríguez-Sáinz MC 13
 Rosenwadj FM 9
 Roucard C 19
 Rovira M 5
 Rubinstein E 8

 Saas P 7, 13, 17
 Sabatier F 1
 Sainty D 4, 11, 14
 Salaun V 14
 Salaün V 14
 Salou M 3
 Samadi M 7
 Sampol J 1
 San Miguel JF 9
 Sánchez-Ramón S 13
 Sangaré M 3
 Sawadogo D 3
 Schrappe M 5
 Servant M 17
 Solary E 6, 7
 Soler F 2
 Sordet O 6
 Sotto JJ 19
 Sudaka I 14

 Tatou E 3
 Teuma X 2
 Trimoreau F 14
 Troussard X 14
 Tupitsyn N 12

 Ugarriza A 19

 Vacher JF 6
 van't Veer M 1
 Viallet A 6
 Vidriales B 9
 Villamor N 2, 5, 19
 Volkova M 12

 Yacoub M 18
 Yseabert L 3

 Zandotti C 11

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