Detection of megakaryocyte colonies in plasma clot cultures by immunoenzymatic staining

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***In vitro* induced megakaryocytic differentiation/maturation of megakayocyte (meg) progenitors represents an important tool for investigating cytokine-induced *in vitro* thrombocytopoiesis.[1-3] We have developed an assay which allows the in situ study of human meg progenitor-derived colonies, cultured on a plasma clot in the presence of cytokines. Plates were immunostained by using an anti-αIIbβ3 monoclonal antibody and an alkaline phosphatase-labeled secondary antibody. αIIbβ3-bearing cells were stained an intense red and were clearly differentiated from the negative cells. Processed plates were stable for some weeks at 4°C. The described procedure is easy to perform and allowed us to enumerate the meg colonies and assess colony morphology and cell ploidy.**

Low density mononuclear cells were isolated from heparinized bone marrow samples by density gradient and adherent cells were removed. A plasma clot assay with modifications was performed as described elsewhere,[4] in the presence of recombinant human cytokines: 100 ng/mL of GM-CSF, IL-3 and IL-6, respectively; or 10 ng/mL of MGDF, IL-3 and IL-6, respectively. In a patient with essential thrombocythaemia, spontaneous growth[5] was observed in the absense of exogenous stimuli.

After 11 or 19 days of incubation, plasma clot was p-formaldehyde-fixed *in situ* for 1 h at 4°C and washed three times at room temperature with 0.1 M phosphate buffered saline solution, pH 7.4 (PBS). Fixed plates were incubated with 1 µg/mL mouse anti-human αIIbβ3 antibody (P2, Immunotech, Marseille, France) for 3h at 37°C or overnight at 4°C. Antibody excess was removed by three washings with 0.05% Tween 20-PBS (TPBS). Alkaline phosphatase (AP)-labelled anti-mouse IgG (ICN, Costa Mesa, CA, USA) was used 1:2000 for 3h at 37°C or overnight at 4°C, followed by three TPBS and one PBS washes. AP activity was detected by incubating plates for 1 h at 37°C with the chromogenic substrate, Fast Red TR/Naphthol AS-MX (Sigma); the reaction was stopped by rinsing with TPBS.

Red colored αIIbβ3-positive cells were clearly identified and distinguished from the negative ones (Figure 1 A-D).

The described procedure is simple and presents a number of advantages in terms of specificity, and reliability, such as:

1) the meg progenitor identification is absolutely specific: in humans, αIIbβ3 integrin (CD41a antigen) is expressed exclusively on megs and platelets:[6] P2 monoclonal antibody reacts with the intact αIIbβ3 complex, but not with αIIb or β3, separately.[7] It should be noted that β3 integrin (CD61 antigen) is a widespread surface marker, also present on monocytes/macrophages and endothelial cells;[8]

2) immunoAP is primarily used for meg progenitor detection in plasma clot cultures,[9] allowing a higher sensitivity than immunoperoxidase and immunofluorescence;

3) the procedure eliminates the need for fluorescent-labelled reagents. Furthermore, since fluores-

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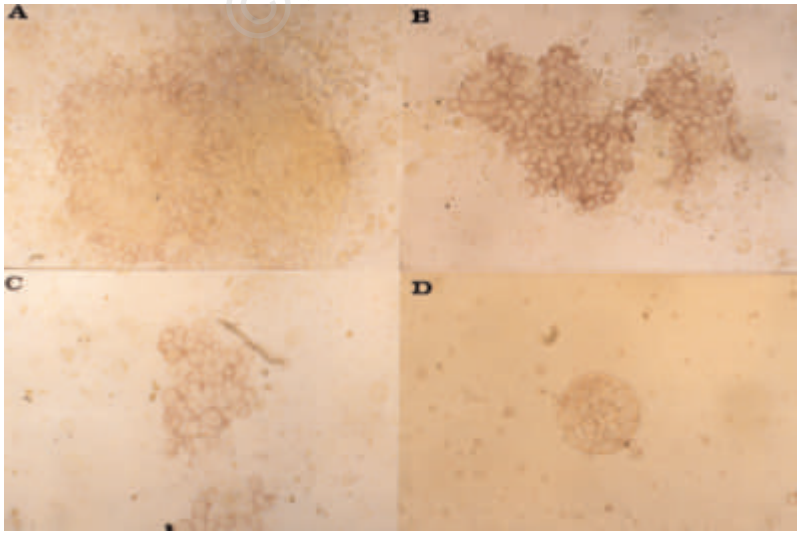

Figure 1. *In vitro* immunostaining of human bone marrow-derived megakaryocyte colonies (original magnification x312).
A: an αIIbβ3-posivite megakaryocyte colony, consisting of small megakaryocytes, closely located to a negative colony.
B-C: megakaryocyte colonies identified by intese red reaction colour in a clean background.
D: a positive polyploid megakaryocyte observed in *in vitro* spontaneous growth from a patient with essential thrombocythaemia (see text). Note the intact cell morphology.

cent emission is subject to decay, plates should be evaluated as soon as possible. Our technique leads to the formation of a stable reaction colour: plates stored at 4°C up to four weeks retained initial staining characteristics;

4) unlike immunofluorescence, the procedure allows the evaluation of meg colonies *in situ* and to contemporarily observe positive, negative, and confluent colonies present in the visual field. Moreover, the technique allows the enumeration of colonies, visualization of intact colony morphology (Figure 1 A-C) and assessment of cell ploidy (Figure 1D). Finally, meg colonies immunoenzymatically identified can be stained further by May-Grünwald-Giemsa for a morphological examination of the single element[10] and offers a permanent record.

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Aggressive natural killer cell leukemia: report of a case in a Caucasian boy

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We report a case of aggressive natural killer cell leukemia (ANKL) in a Caucasian boy diagnosed on clinical, cytologic, immunophenotypic and genotypic grounds. An anomalous karyotype and integration of Epstein-Barr virus (EBV) within the leukemic population were identified.

Large granular lymphocytes (LGL) are a morphologically recognizable lymphoid subset comprising 10% to 15% of peripheral blood mononuclear cells. CD3$^-$ LGL are natural killer (NK) cells that mediate nonmajor histocompatibility complex (MHC)-restricted cytotoxicity and do not express the CD3/T-cell receptor (TCR) complex or rearrange TCR genes. They show variable expression of the NK cell markers CD16, CD56 and CD57. CD3$^+$ LGL are T cells that do express the CD3/TCR complex, often with expression of one or more NK cell markers, and rearrange TCR genes. These cells are cytotoxic T lymphocytes that can mediate nonMHC-restricted cytolysis.[1]

Generally, LGL leukemia is divided into two major categories based on the surface phenotype, T cell type and NK cell type.[2] Aggressive NK cell leukemia (ANKL) was first described by Inamura *et al.* in 1990.[3] These researchers reported their cases as aggressive NK cell leukemia/lymphoma but in the recent classification of NK cell and NK-like T-cell malignancies proposed by Jaffe,[2] a distinction is made between ANKL cases and those considered as NK lymphomas (angiocentric lymphomas), which usually present with localized extranodal disease (nasal and nasal-type NK cell lymphomas). Although there are sporadic reports of ANKL,[3-9] mainly from Japan, little is known about the pathogenesis of this disease and no recurrent clonal chromosomal abnormality has been described to date.

A 16-year-old Caucasian boy was admitted to the hospital for malaise and jaundice. Physical examination and computerized axial tomography revealed marked hepatosplenomegaly but no lymphadenopathies.

Initial blood count showed a hemoglobin level of 11.9 g/dL, platelet count 19×10^9/L and white blood cell count 12.69×10^9/L with 18% circulating atypical lymphocytes. The biochemistry profile included serum γ-glutamyl transpeptidase (γ-GTP)

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78 IU/L (normal, 8 to 38 IU/L), glutamic pyruvic transaminase (GPT)140 IU/L (normal, 0 to 29 IU/L), glutamate oxaloacetate transaminase (GOT) 380 IU/L (normal, 0 to 25 IU/L), bilirubin 11.27 mg/dL (normal, 0 to 1 mg/dL), conjugated bilirubin 7.74 mg/dL (normal, 0 to 0.25 mg/dL), lactic dehydrogenase (LDH) 2540 IU/L (normal, 160 to 320 IU/L), triglycerides 330 mg/dL (normal, 70 to 150 mg/dL), proteins 5.79 g/dL (normal, 6.6 to 8.7g/dL). Other laboratory data were within the normal range. Coagulation profile was: prothrombin time (PT) 55%, activated partial thromboplastin time (APTT) 53 seconds (27.9"), fibrinogen 1. 3 g/L (normal, 2 to 4 g/L), D-dimer 1500 ng/mL (normal, < 500 ng/mL). A bone marrow (BM) biopsy showed interstitial infiltration by atypical lymphocytes. A cavum biopsy and cerebrospinal fluid examination were normal. The surface phenotype of the peripheral blood (PB) and BM lymphocytes was $CD1^-$, $CD2^+$, $CD3^-$, $CD4^-$, $CD5^-$, $CD7^+$, $CD8^+$, $CD25^+$, $CD38^+$, $CD56^+$, $CD57^-$.

A diagnosis of non-Hodgkin´s T lymphoma in leukemic phase with liver dysfunction was formulated and the patient was started on treatment with cyclophosphamide, followed by a course of combination chemotherapy including cyclophosphamide, adriamycin, vincristine, prednisone (CHOP therapy) and granulocyte colony-stimulating factor (G-CSF). Due to the lack of a clinical response, treatment was changed to a regimen containing ifosfamide (with MESNA), etoposide and mitoxantrone, and subsequently to an induction protocol for lymphoblastic leukemia with vincristine, daunoblastin, dexamethasone and asparaginase.

The disease progressed shortly afterwards and three new courses of ifosfamide, etoposide, mitoxantrone, dexamethasone and G-CSF were given. In the absence of a clinical response, the patient was referred to our hospital. On examination the patient appeared to be severely ill. He had prominent hepatosplenomegaly without peripheral adenopathies. Hemogram revealed a hemoglobin concentration of 8.5 g/dL, platelets 22×10^9, leukocytes 7.81×10^9 with 50% circulating atypical cells. BM atypical cells accounted for 50% of all nucleated cells. These cells were extremely variable in size, ranging from small lymphoid cells with clumped chromatin to large cells with a more blast-like appearance, open chromatin and nucleoli. Most cells presented a low nucleus/cytoplasm ratio and intense basophilic cytoplasm containing azurophilic granules (Figure 1). Flow cytometric immunophenotyping was performed using a direct immunofluorescence technique. Samples were analyzed on a FACScan flow cytometer [Becton Dickinson (BD), San José, CA, USA] using a LYSIS II and PAINT-A-GATE software programs. Atypical cells expressed the following phenotype: $CD2^+$ [Coulter Corp., (C) Hialeah, Florida, USA], $CD7^+$ (BD), $CD8^+$ (BD), $CD16^+$ (BD), HLA-DR^+ (BD), $CD38^+$ (BD), $CD56^+$ (BD), $CD94^+$ (HP-3B1 kindly provided by Dr. López-Botet, Hospital de la Princesa, Madrid), cytoplasmic $CD3^+$ (BD), surface $CD3^-$ (BD), $CD4^-$ (BD), $CD5^-$ (BD), $CD10^-$ (BD), $CD11b^-$ (BD), $CD13^-$ (BD), $CD14^-$ (BD), $CD19^-$ (C), $CD21^-$ [Serotec Ltd, (ST) Oxford, England], $CD22^-$ (ST), $CD25^-$ (BD), $CD33^-$ (BD), $CD34^-$ (ST), glycophorin A^- [Immunotech, (I) Marseille, France], $CD36^-$ (I), $CD41^-$ (ST), $CD61^-$ (BD), $CD57^-$ (BD), $\alpha\beta$ TCR^- (ST), $\gamma\delta$ TCR- (T cell diagnostics Inc, Cambridge, England), nuclear TdT^- (Sera-Lab Ltd, Sussex, England). Cytogenetic study determined the karyotype to be 46, XY/46, XY, 6q–, –7 , +mar, after short-term culture without stimulation and G-band-

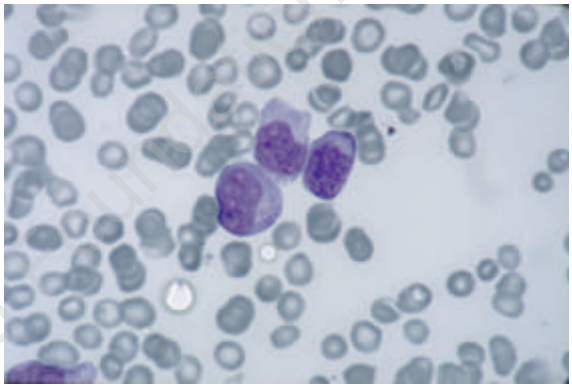

Figure 1. Peripheral blood smear (May-Grünwald Giemsa, ×1000) showing atypical lymphoid cells with basophilic cytoplasm and azurophilic granules.

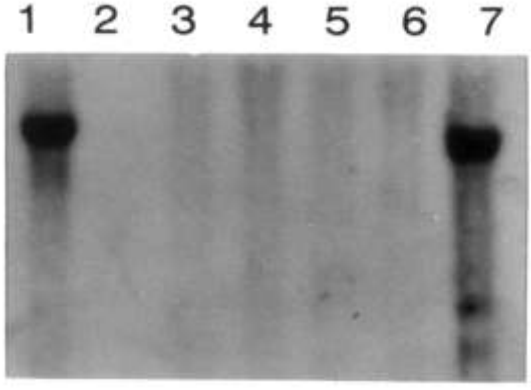

Figure 2. Southern Blot analysis of Epstein-Barr virus (EBV) termini. Identification of one band of 8.7 kb on BamHI digests with a probe to the terminal repeat region of EBV shows that the case (lane 1) contains a significant amount of homogeneous episomal EBV DNA indicative of a clonal population. Lane 7 represents a single 7.4 kb band detected in a non-Hodgkin lymphoma (NHL) from a HIV-positive patient. All other lanes corresponded to other NHL; none of these hybridized with the EBV probe.

ing. The TCR-β gene was found to be in the germline configuration after analysis using a Cβ on BamHI and EcoRI digests.[10] Southern blot analysis of EBV termini showed the presence of one 8.7 kb band on BamHI digests with a probe specific for the EBV genomic termini[11] (Figure 2). A diagnosis of ANKL was formulated, but the patient died shortly afterwards due to multiorgan failure, 5 months after diagnosis.

A diagnosis of ANKL in our patient is supported on clinical, morphologic, immunophenotypic and genotypic grounds. Clinically, the entity is characterized by hepatosplenomegaly with disseminated disease, BM and PB involvement and an aggressive clinical course.[2-8]

In our case, atypical lymphocytes were $CD2^+$ and expressed the NK markers CD56 , CD16 and CD94. They did not express surface CD3, CD5, αβ TCR, or γδ TCR and presented TCR in the germline configuration. These findings argue against a T-cell lineage and strongly support NK lineage. On the other hand, CD8 and cytoplasmic CD3 expression (Leu4) have been reported more frequently in T-cell malignancies. Nevertheless, expression of both antigens has been described in cases considered to be of NK lineage.[9,13] In fact, a subset of normal NK cells expresses CD8α,[14] and although adult NK cells only express cytoplasmic CD3ϵ, fetal NK cells express cytoplasmic CD3ϵ, CD3γ and CD3δ,[14] a complex that is recognized by the leu4 antibody. On the basis of leu4 positivity and TCR gemline configuration, some authors have hypothesized that the cellular origin of the malignant population might correspond to these fetal NK cells.[13] However, since there is little information available regarding the use of flow cytometry for the study of cytoplasmic CD3 in NK cell malignancies, we could not ascertain the true origin of these cells.

The clonal nature of the NK cell proliferation was demonstrated by the finding of a single band in Southern blot analysis using the EBV genomic termini probe, and by the detection of an aberrant karyotype. Although the association of EBV and ANKL is variable,[2] the demonstration of clonal EBV episomal DNA in the neoplastic population raises the possibility of an etiological role for EBV in the leukemic transformation, as has been suggested by some authors.[9] In contrast with the finding of an anomalous karyotype and clonal integration of EBV in our case and in other aggressive cases, mainly in Japanese patients,[4] some studies in European and American patients with chronic $CD3^-$ LGL lymphocytosis were unable to demonstrate clonality or the presence of the EBV genome.[4] Nevertheless, as noted above, progression from a chronic form to an aggressive phase has been previously reported. Further studies are thus needed to determine in which way EBV could contribute to the neoplastic transformation and to the aggressive course of the disease.

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