The flow cytometric pattern of CD34, CD15 and CD13 expression in acute myeloblastic leukemia is highly characteristic of the presence of PML-RARα gene rearrangements

ABSTRACT

Background and Objective. Rapid identification of AML patients carrying the t(15;17) translocation for treatment decision-making is currently made on the basis of morphologic screening. However, the existence of both false positives and negatives highlights the need for more objective methods of screening AML cases and further molecular confirmation of the t(15;17) translocation.

Design and Methods. In the present study we analyzed a total of 111 AML cases in order to investigate whether immunophenotyping based on the assessment of multiple stainings analyzed at flow cytometry could improve the sensitivity and specificity of morphologic identification of acute promyelocytic leukemia (APL) carrying the t(15;17) translocation. FISH analysis was used as a complementary technique for cases in which morphology and molecular biology yielded discrepant results.

Results. Concordant results between morphology and RT-PCR were found in 102/111 (91.8%) cases: 34 patients had M3/PML-RARα+ and 68 non-M3/PML-RARα– disease. Nine cases showed discrepant results. Multivariate analysis showed that the best combination of immunologic markers for discriminating between M3/PML-RARα+ and non-M3/PML-RARα– cases was that of the presence of heterogeneous expression of CD13, the existence of a single major blast cell population, and a characteristic CD34/CD15 phenotypic pattern (p<0.02). A score system based on these parameters was designed, and the 34 M3/PML-RARα+ cases showed a score of 3 (presence of the 3 phenotypic characteristics). In contrast, only 1 out of the 68 (1.3%) non-M3/PML-RARα– cases had this score, most of these latter cases (53/68, 78%) scoring either 0 or 1. Therefore, among these cases, immunophenotyping showed a sensitivity of 100% and a specificity of 99% for predicting PML/RARα gene rearrangements.

Of the 9 cases in which morphology and molecular biology results were discrepant, four cases displayed M3 morphology without PML/RARα rearrangements by RT-PCR. In only one of these 4 cases did the immunophenotype score 3, this being the only FISH positive case. From the remaining five discrepant cases (non-M3 morphology while positive for PML/RARα) two cases had a phenotypic score of 3 and were FISH positive while the other three were negative by FISH. Upon repeating RT-PCR studies, two of these latter three cases became negative.

Interpretation and Conclusions. Our results show that immunophenotyping may be of great value for quick screening of APL with PML/RARα rearrangements.

Key words: acute myeloblastic leukemia, t(15;17)
itive diagnoses and false negative results. Additionally, although t(15;17) translocation is one of the most frequent structural chromosome abnormalities in AML patients it is only present in a relatively low proportion of AML cases, its incidence ranging from 6% up to 38% depending on the geographical area.10 Because of this relatively low incidence, molecular confirmation of the PML/RARα gene involvement is not generally investigated in all AML cases but on the select cohort of patients that are morphologically diagnosed as having M3 leukaemias.1,3,4,6,11,12 Accordingly, the availability of other more objective methods of screening APL leukaemias for further molecular confirmation would be of great value.

For more than 10 years a clear association between FAB M3 morphology and the immunophenotype of AML blast cells has been established. Blast cells in APL usually co-express the myeloperoxidase (MPO), CD9, CD13 and CD33 myeloid-associated markers in the absence of reactivity for HLA-DR.13,14 In spite of this association, AML patients displaying this phenotype still form a heterogenous group of patients including a substantial proportion of patients who do not display the t(15;17) translocation; moreover, a small proportion of APL cases are HLA-DR+.13,16 Due to these limitations immunophenotyping of AML blast cells has been considered as a secondary diagnostic tool with respect to morphology for the identification of cases carrying the t(15;17) translocation and its use has been almost exclusively restricted to supporting the morphologic diagnosis of M3 variants (hypogranular morphology).17-19 In recent years the availability of antibody reagents directed against the PML protein has shown that both the wild PML protein and the PML-RARα fusion protein display different immunocytochemical patterns of staining which directly correlate with the molecular studies.20-22 However, discrepant results have been reported regarding the characteristic intracellular distribution of the fused nuclear21,22 versus cytoplasmatic20-23 protein. Moreover, Falini et al.22 showed that in up to 8% of the AML cases analyzed no staining was observed with the anti-PML monoclonal antibody, these patients being considered as false negative cases with respect to the expected PML reactivity.

In parallel to reagent development important advances have occurred in recent years regarding the immunophenotypic characterization of leukemic cells as a result of the possibility of performing multiple stainings which allow the identification of leukemic cells by distinguishing them from their normal counterparts, and the specific analysis of leukemic cell differentiation.18,19,24 In spite of this, to the best of our knowledge no study has been performed in which the sensitivity and specificity of multiparametric immunophenotyping has been compared with conventional morphologic criteria for the identification of cases carrying the t(15;17) translocation.

The aim of the present study was to evaluate the immunophenotypic characteristics of AML patients based on multiple stainings analyzed by flow cytometry in order to assess whether or not immunophenotyping improves the sensitivity and specificity of morphology and provides a useful tool for the screening of cases in which the t(15;17) translocation should be systematically searched for by molecular techniques.

**Design and Methods**

**Patients**

A total of 111 patients (104 adults and 7 children) whose BM samples were stored in the reference laboratory of the University Hospital of Salamanca were included in the present study. All patients had an unequivocal diagnosis of de novo AML based on morphologic, cytochemical25 and immunophenotypic criteria.17 Sixty-eight patients were male and 43 were female with a mean age of 51±21 years; the range was from 6 to 87 years old. Two experts independently assessed FAB morphology. All cases were studied at diagnosis.

**Immunophenotyping studies**

In all cases immunophenotyping studies were performed at diagnosis on erythrocyte-lysed whole bone marrow (BM) samples upon staining with monoclonal antibodies directly conjugated with fluorochromes. Antigen expression was analyzed on a FACSort flow cytometer (Becton Dickinson, San José, CA, USA) using double and triple-stainings with the following combinations of fluorochrome-conjugated monoclonal antibodies (fluorescein isothiocyanate [FITC], phycoerythrin [PE] and either peridinin chlorophyll protein [Per-CP] or the PE/cyanide 5 [Cy5] fluorochrome tandem) directed against surface antigens: CD15/CD117/CD34, CD15/CD33/CD34, CD15/CD34/HLA-DR, CD34/CD38/CD19, CD34/CD56/CD33, HLA-DR/CD33/CD13, CD7/CD13/CD19, CD65/CD11b/CD4, CD2/CD14/CD13, CD61/glycoporphin A/CD45, CD10/CD5/CD20 and CD71/CD11b. In addition the expression of MPO, CD79a and CD3 was also explored at the cytoplasmic level.

Briefly, BM samples were obtained and immediately diluted in phosphate buffered saline (PBS) containing K3 EDTA as anticoagulant in a 1/1 (vol/vol) proportion. Afterwards, for surface antigenic stainings, 200 µL of PBS-diluted BM samples, containing between 0.5 and 1×10⁶ nucleated cells were placed in each tube and incubated with the appropriate combination of monoclonal antibodies for 10 minutes in the dark (room temperature). Once this incubation period was finished, 2 mL of FACS lysing solution (Becton/Dickinson) diluted 1/10 (vol/vol) in distilled water were added to each tube and after vigorous vortexing another incubation for 10 minutes in the dark (room temperature) was performed. Cells were then centrifuged (5 minutes at 540 g), washed once in 2 mL of PBS/tube (5 minutes at 540 g) and resuspended in 0.5 mL/tube of PBS.
For the staining of cytoplasmic antigens (MPO, CD79a and CD3) the Fix & Perm reagent from Caltag Laboratories (San Francisco, CA, USA) was used. Briefly, 50 µL of sample were incubated with 100 µL of solution A from the Fix & Perm reagent for 15 minutes (room temperature). Afterwards, cells were washed once in 2 mL of PBS/tube and resuspended in 100 µL of an erythrocyte-lysing, leukocyte-permeabilizing solution (solution B from the Fix & Perm reagent). In addition, 10 µL of anti-MPO-FITC, anti-CD79a-PE and anti-CD3-PE/Cy5 were added and cells incubated for another 15 minute period at room temperature. Afterwards, cells were resuspended in 2 mL of PBS/tube, centrifuged (5 minutes at 540 g) and resuspended in 0.5 mL of PBS.

The source and specificity of each monoclonal antibody used in the present study was as follows: CD34 (HPCA-2-PE, My10-FITC and HPCA-2 PerCP), CD15 (leu M1-FITC ), CD33 (leu M 9-FITC), HLA-DR (anti-HLA DR-PerCP and anti-HLA DR-FITC), CD38 (leu 17-PE), CD56 (CD158-PE), CD13 (leu 19-PE), CD7 (leu 9-FITC), CD2 (leu 5b-FITC), CD11b (leu 15-PE), CD14 (leu M 3-PE), CD45 (HL E-1-PerCP), CD10 (CALLA-FITC), CD5 (leu 1-PE), CD71 (anti-transferin receptor-FITC) and CD3 (leu 4-PerCP), were purchased from Becton/Dickinson; CD117 (95C3-PE) and glycophorin A (D2.10-PE) from Immunotech (Marseille, France); CD19 (SJ25C1-PE/Cy5 ), CD33 (4D3-PE-Cy5), CD13 (TUK 1-PE-Cy5), CD65 (VIM 2-FITC), CD4 (53.5-PE/Cy5), M PO (H435-FITC) and CD20 (H147-PE-Cy5) were obtained from Caltag Laboratories and CD61 (Y2/51-FITC) and CD79a (HM 57-PE) from Dako (Glostrup, Denmark).

Isotype-matched mouse non-specific immunoglobulins and a tube stained for the CD3-FITC, CD4-PE and CD8-Pe-Cy5 antigens were used as negative and positive controls, respectively.

Data acquisition was performed on a FACSort flow cytometer (Becton/Dickinson) using the LYSYS II software program (Becton/Dickinson). A minimum of 15,000 events/tube from the total BM cellularity were acquired. In order to make results comparable between different days, careful instrument calibration and fluorescence compensation was performed using both CALIBRITE beads (Becton/Dickinson) and normal PB lymphocytes stained for CD3-FITC, CD4-PE and CD8-PE/Cy5 as described elsewhere.26 The PAINT-A-GATE PRO software (Becton/Dickinson) was used for data analysis. Whenever necessary, further stainings were made in a second step in order to obtain specific information on the phenotypic characteristics of the leukemic cells. The following information was specifically explored in leukemic cells for each of the antigens analyzed: presence or absence, fluorescence intensity and pattern of expression (homogeneous versus heterogeneous). A pattern of antigen expression was defined as heterogeneous if the cells occupied more than one logarithmic decade on the scale of fluorescence intensity. The presence of two or more major blast cell subpopulations was defined on the basis of the existence of phenotypically different leukemic cell subsets which each represented more than 25% of all neoplastic cells for any of the antigens analyzed except CD34.26

**PCR amplification of PML/ RARα transcripts**

RNA was extracted from washed BM mononuclear cells by the guanidium thiocyanate method of Chomczynski and Sacchi.27 Reverse transcription (RT) was performed on 1 µL of total RNA, after heating at 70°C for 10 minutes with random hexamers as the reaction primer. The reaction was carried out at 42°C for 1h in a 20 µL volume containing 200 U of Superscript II (Life Technologies Incnhinan, Scotland, UK) according to the manufacturer’s instructions. Subsequently, 5 µL of RT products were used for two-step PCR analysis according to the guidelines proposed by Biondi et al.28 PCR conditions were as follows: reaction volume of 100 µL containing 1.5 mmol/L MgCl2, 50 mmol/L KCl, and 10 mmol/L Tris HCl; pH 8.8; 200 µmol/L dNTP, 2.5 U of Taq DNA polymerase and 30 pmol of each primer. Primers used were M 4, M 2, R5 and R8, as previously described.28

PCR was performed in a GeneAmp PCR System 9600 thermocycler (Perkin Elmer, Foster City, CA, USA). After an initial denaturation at 95°C for 5 minutes, denaturation, annealing and extension were carried out at 95°C for 1 minute, 55°C for 1 minute and 72°C for 1 minute, respectively, for a total of 30 cycles, with the last extension at 72°C lasting for 10 minutes. The first PCR was performed with the M 4 primer (bcr 3 breakpoint) or M2 (bcr 1 or bcr 2 breakpoint) as 5’ primers and the R5 as 3’ primer. For the second-round PCR, the system used was the same as for the first round except that 5 µL of the first-PCR product were used instead of the RT product and the substitution of R5 primer for the nested primer (R8). Finally, 20 µL of the PCR product were electrophoresed on a 2% Nu Sieve agarose gel stained with ethidium bromide and visualized under UV light. Two negative controls (one with non-APL RNA and one without RNA) and one positive control APL sample were included in each experiment. The integrity of the RNA preparation was assessed by amplification of normal RARα as previously described by Borrow et al.29 In order to exclude false positive or negative results, those samples with discrepancies between RT-PCR and morphology underwent to new RT-PCR assays using a different whole BM aliquot.

**FISH studies**

In those de novo AML cases in which discrepant results were found between morphology and the RT-PCR techniques, FISH analysis for the t(15;17) translocation was performed. For that purpose the LSI-PML/RARα dual color probe (Vysis Inc., Downers
Grove, IL, USA) was used. The PML probe stained with spectrum orange begins in intron 7, a span of 5.8 Kb and extends centromerically on chromosome 15 approximately 180 Kb. The RARα probe begins less than 6 Kb 3’ to intron 2 and extends approximately 400 Kb telomerically on chromosome 17. Accordingly, the combination of both probes covers the three break-point clusters (bcr 1, bcr 2 and bcr 3) within the region of 13 Kb of the PML gene on chromosome 15.

Prior to hybridization 1 µL of probe solution (Vysis) was mixed with 7 mL of LSI hybridization buffer (Vysis) and 2 µL of distilled water, centrifuged for 2 seconds and heated for 5 minutes in a 74±1°C water bath for denaturing purposes.

FISH analysis was performed on cells from BM samples prepared according to conventional cytogenetic techniques. Briefly, the slides containing fixed cells were immersed in a denaturing bath (70% for-mamide in 2x SSC buffer placed at 74±1°C) for 5 minutes and then dehydrated according to previously reported techniques7,8 and dryed. Afterwards, slides were warmed to 45-50°C for 2 minutes and 10 µL of the denaturated probe-mix was applied to each slide. Hybridization was then allowed to take place by incubating the slide overnight (16 hours) in a pre-warmed humidified box placed in a 37°C incubator. Once this incubation period was finished, slides were washed 3 times (3×10 minutes) in 50% formamide in 2× SSC (pH =7.0) at 46°C. Another wash for 10 minutes in 2× SSC (pH =7.0) was then performed at room temperature. Finally, the slides were washed for 5 minutes (room temperature) in 2× SSC containing 0.1% of Tween 20 (Sigma, St. Louis, MO, USA). The slides were then allowed to dry and 10 µL of a counterstaining solution containing 75 ng/mL of DAPI (Sigma) and 20 mg/mL of 1,4-diazobicyclo-2,2,2-octane (Sigma) used as an antifading agent were added to each slide.

Fluorescence signals were evaluated using a DM RX fluorescence microscope (Leica, Wetzlar, Germany) and a minimum of 200 cells/sample were analyzed including both interphase nuclei and metaphases. For all slides measured the number of unhybridized cells in the areas assessed was lower than 1% and only those spots with similar size, intensity and shape were counted. Cells with fusion or juxtaposed green and red signals were interpreted as positive for t(15;17).

Cells with fusion or juxtaposed green and red signals were interpreted as positive for t(15;17). The slides were then allowed to dry and 10 µL of a counterstaining solution containing 75 ng/mL of DAPI (Sigma) and 20 mg/mL of 1,4-diazobicyclo-2,2,2-octane (Sigma) used as an antifading agent were added to each slide.

Fluorescence signals were evaluated using a DMRX fluorescence microscope (Leica, Wetzlar, Germany) and a minimum of 200 cells/sample were analyzed including both interphase nuclei and metaphases. For all slides measured the number of unhybridized cells in the areas assessed was lower than 1% and only those spots with similar size, intensity and shape were counted. Cells with fusion or juxtaposed green and red signals were interpreted as positive for t(15;17).

Statistical methods

The relative frequencies of all the phenotypic variables included in the present study were calculated. Comparisons between groups were performed using the chi-square test and p values lower than 0.01 were considered to be associated with statistically significant differences (SPSS 5.0 Inc., Chicago, IL, USA).

For the assessment of the power of immunophenotypic criteria for discrimination between APL and non-APL cases (based on the presence or absence of PM L/RARα transcripts) multivariate analysis was performed using a logistic regression model with the forward stepwise option and a probability comparison test (SPSS 5.0 Inc.). The immunophenotypic variables included in the multivariate analysis were those displaying statistical significant differences in the univariate study.

Results

Of the 111 de novo AM L cases included in the present study 38 displayed an M3 morphology (34 cases were typical M3 and four were considered hypogranular M3 variants). Of the remaining 73 cases, 9 corresponded to M0, 13 to M1, 19 to M2, 10 to M4, 6 to M4Eo, 8 to M5a, 4 to M5b and 4 to M6. Initially, molecular studies showed the presence of the PM L/RARα RNA transcript in a total of 39 out of the 111 patients (35%). Of them, 34 corresponded to AM L cases with an M3 morphology, and the remaining 5 cases were classified as having M0 (2 cases), M1 (one case) and M2 (2 cases) leukemias. Accordingly, four different groups of patients could be established on the basis of the results obtained with both morphologic and molecular biology techniques. In the two major groups there was concordance between both methods: 1) M3 cases being PM L/RARα+ (n=34); and 2) non-M3/PML-RARα+ cases (n=68); the remaining two groups included cases in which morphology and RT-PCR studies showed discrepant results; 3) M3 morphology with negativity for the PM L/RARα transcripts (n=4); and 4) non-M3/PML-RARα+ AM L cases (n=5). RT-PCR studies were repeated in all the cases from these latter two groups of patients confirming the initial findings in all cases except for one M0 and one M1 patient who were initially PM L/RARα+ but became negative in the second and third analyses.

For the analysis of the immunophenotypic characteristics of the 111 AM L patients, we first divided the series into two groups: the M3/PML-RARα+ and non-M3/PML-RARα+ cases (Table 1). In the former group the leukemic blast cells showed the following common characteristics: 1) homogeneous expression of CD33-PE in all blast cells (82% of the cases) (Figure 1B); 2) reactivity for CD13-PE in all leukemic cells (82% of cases) (Figure 1C); 3) a singular pattern of expression for the CD34-PE/CD15-FITC antigens in which leukemic cells lose CD34 before they acquire CD15 expression, and the blast cells never acquire high levels of CD15 (100% of the cases) (Figure 1D and E); 4) absence of reactivity for HLA-DR-FITC (91% of the patients) (Figure 1B) and 5) presence of a single major blast cell population which may be defined by these antigens (100% of the cases). Interestingly, in the non-M3/PML-RARα negative cases the incidence of each of the five immunophenotypic characteristics mentioned above was significantly lower (p<0.00001) (Table 1).

Multivariate analysis showed that the best combi-
Immunophenotype of AML with t(15;17)

The immunophenotypic characteristics of M3/PML-RARα+ and non-M3/PML-RARα- AML cases were studied using a combination of univariate and multivariate analyses. The characteristics included the typical CD34/CD15 pattern (100% vs. 21%, p<0.00001), the expression of CD13 (100% vs. 34%, p<0.00001), and the presence of one major blast cell subset (100% vs. 56%, p<0.00001). These characteristics were used to develop a score system that was applied to two major groups of AML patients: M3/PML-RARα+ and non-M3/PML-RARα-. The score system revealed that all 34 M3/PML-RARα+ cases had a score of 3, while only 1 out of 68 non-M3/PML-RARα- cases had this score. Most of the other cases (53/68, 78%) scored either 0 or 1. Therefore, immunophenotyping had a sensitivity of 100% and a specificity of 99% for predicting PML/RARα gene rearrangements. Within the 9 cases in which morphology and molecular biology were initially discrepant, four cases displayed M3 morphology without PMI/LRARα gene rearrangements by RT-PCR (Table 2). In only one of these 4 cases was the score 3. Of the five cases with non-M3 morphology which were PMI/LRARα+, two cases had a phenotypic score of 3 (Table 2). Table 3 shows the results obtained with the FISH technique for the analysis of the t(15;17) translocation in all these 9 cases with discrepant morphologic and molecular findings. As shown in it, FISH studies were positive only in those three cases with an immunophenotypic score of 3 while the remaining 6 cases were negative. As mentioned earlier, in all these 9 cases molecular biology studies using RT-PCR were repeated using different aliquot samples. In these repeated experiments, only three out of the 5 non-M3/PML-RARα+ patients remained constantly positive while the other two cases became negative. These latter two cases were patients with an immunophenotypic score of 1 (Table 3). In contrast, the four M3/PML-RARα+ cases were repeatedly negative for the PML-LRARα transcript, but one of them, with a score of 3 by immunophenotyping, showed a rearranged RARα gene by Southern blot analysis.

Discussion

At present, BM morphology is still commonly used as the only diagnostic tool for treatment decision-making in APL although the accuracy of morphology...
is considered to be insufficient. Thus, demonstration of a PM-L-RARα rearrangement as a result of the t(15;17) translocation is mandatory due to the sensitivity of APL to ATRA treatment.1-6 As a matter of fact, in a recent study by Tallman et al.12 showed that 40% of the cases that were diagnosed as M 3 leukemia and in which ATRA treatment was started were then shown to be PM-L-RARα, which would correspond to false positive cases by morphology, and subsequently these patients, had to interrupt ATRA treatment. In a Spanish trial, the reported incidence of false positive APL based on morphology (M 3) was 14%.11 To the best of our knowledge, few studies using molecular analysis have been performed to assess the incidence of false negative cases by morphology, in order to exclude, among the non-M 3 leukemias, the presence of some PM-L-RARα, also showing the existence of false negative cases by morphology.

In the present study, the simultaneous assessment of BM morphology and the PM-L-RARα translocation in a group of 38 M 3 leukemias and 73 consecutive AML cases with a non-M 3 morphology showed that, although in most cases (91.8%) agreement is observed between both methods, there is also a small proportion of M 3 cases (10.5%) which do not have the PM-L-RARα transcript, as well as cases with non-M 3 morphology that are PM-L-RARα+ based on diagnosis by RT-PCR techniques (6.8% of all the non-M 3 cases). This supports the notion22 that not only false positive but also false negative cases may exist if morphology is taken as the single tool for treatment decision making and to select cases who are candidates for further molecular studies.

For a long time APL has been shown to display a characteristic phenotype which has been mainly associated with the co-expression of pan-myeloid markers such as CD13 and CD33 in the absence of reactivity for the HLA-DR antigen. However, both HLA-DR+ APL and HLA-DR- AML displaying morphologic characteristics distinct from those of M 3 leukemias have been shown to exist, which suggests that immunophenotyping has a limited value as a primary diagnostic tool in APL.17-19,31

However, nowadays, the immunophenotypic characterization of blast cells should no longer be based merely on the presence or absence of an antigen, since multiparametric analysis using multiple simultaneous stainings analyzed by sensitive flow cytometric methods also provides information on the pattern of antigen expression and may facilitate the identification of particular phenotypic profiles which allow a more comprehensive characterization of leukemic cells. Thus, we have shown that a high proportion of AML patients display leukemia-associated phenotypes, which are distinct from normal progenitor myeloid cells. A possible explanation for these phenotypic aberrations displayed by leukemic cells might be the presence of genetic abnormalities that alter the normal pattern of surface antigen expression. In this hypothesis, specific genetic alterations would be reflected by the existence of characteristic phenotypic features. Accordingly, the aberrant expression of different markers on AML blast cells has been associated with specific genetic abnormalities such as inv16, t(8;21) and t(15;17) and others.18-24 Based on these findings, we explored whether a particular immunophenotype could be specific and sensitive enough to be of utility in the initial screening of cases with PM-L-RARα gene rearrangements in order to have a rapid tool, complementary to morphology, for diagnosis of APL which would increase the accuracy of the treatment decision-making process. For this purpose we used a large panel of monoclonal antibodies in double and triple-staining combinations and a similar approach to that currently used for the investigation of phenotypic aberrations to detect minimal residual disease in AML patients. Accordingly, the immunophenotypic characterization of AML was performed after the specific identification of leukemic blast cells. In addition, for each antigen, information was obtained not only on its presence/absence but also on its pattern of expression (i.e. fluorescence intensity, homogeneity/heterogeneity). Using this strategy we...
found that APL cases with an M<sub>3</sub> morphology and PM-L-RAR<sub>x</sub> translocation displayed characteristic features such as a homogeneous expression of CD33-PE and a heterogeneous reactivity for CD13-PE (both markers being positive in all leukemic cells) together with a characteristic differentiation pattern for the CD34-PE/CD15-FITC antigens. In addition, most cases were HLA-DR negative, as previously described, and displayed a single major blast cell subpopulation for all the antigens explored except CD34. Multivariate analysis showed that the pattern of CD34/CD15 and CD13 expression together with the number of major blast cell subsets was the best combination of variables for distinguishing between M<sub>3</sub>/PM-L-RAR<sub>x</sub> and non-M<sub>3</sub>/PM-L-RAR<sub>x</sub> AML cases. Indeed, the sensitivity of immunophenotyping for selecting PM-L-RAR<sub>x</sub> cases was 100% and the specificity 99%.

The subsequent step was to explore the immunophenotype of the AML cases in which discrepant results were produced by morphology and molecular biology techniques. Our results showed that in 3 out of the 4 M<sub>3</sub>/PM-L-RAR<sub>x</sub>-negative cases immunophenotyping supported the molecular findings, while in the fourth patient the immunophenotype was in agreement with an M<sub>3</sub> morphology but not with the absence of PM-L-RAR<sub>x</sub>. FISH studies supported the immunophenotypic findings in all these 4 discrepant cases since the presence of PM/L/RAR<sub>x</sub> was confirmed by FISH analysis on metaphase and interphase cells in only the fourth case mentioned above. Moreover, although confirmatory PCR was repeatedly negative in this case, Southern-blot analysis showed the existence of a RAR<sub>x</sub> gene rearrangement at the DNA level. Among the 5 non-M<sub>3</sub> cases which were initially PM-L-RAR<sub>x</sub> immunophenotypically supported the molecular diagnosis in two patients, who at the same time, were positive for the (t(15;17)) by FISH. By contrast, immunophenotyping supported the molecular logic results in the remaining three cases none of which showed the (t(15;17)) by FISH. After repeating molecular biology studies, two of these last three cases were shown to be PM-L-RAR<sub>x</sub> negative thus being considered as false positive cases at the initial PCR, presumably due to sample contamination. In fact, as mentioned above, FISH studies confirmed the absence of the (t(15;17)) in these two patients. Thus, a discrepancy remained between PCR(+) and morphology (M<sub>3</sub>), FISH(-) and immunophenotype (score 2) in one case (case 21303). Further studies are necessary in order to elucidate the reasons underlying such discrepancies.

In summary, our results show that in newly diagnosed acute promyelocytic leukemia (APL) validation of short term effect in a large multicenter trial (APL 93 Trial) and assessment of long term benefit (APL 91 Trial) [abstract]. Blood 1996; 88: (Suppl 1): 209a.


