Leukemia-Associated Changes Identified by Quantitative Flow Cytometry. IV. CD34 Overexpression in Acute Myelogenous Leukemia M2 With t(8;21)

By Anna Porwit-MacDonald, George Janossy, Kamal Ivory, David Swirsky, Rowayda Peters, Keith Wheatley, Helen Walker, Alev Turker, Anthony H. Goldstone, and Alan Burnett

During the immunodiagnosis of 517 cases of acute myelogenous leukemia (AML) entered into the Medical Research Council (MRC) AML10 trials, we have observed the CD34 precursor cell antigen more frequently in AML of M2 morphology, especially in the 84% of cases with the t(8;21) chromosome translocation, than in any other French-American-British classification group. CD34 expression was then quantified using QlFl and Quantum Simply Cellular beads (Flow Cytometry Standards, Research Triangle Park, NC) and CD34+ standard cells). When CD34 antibody-binding capacity (ABC) of normal bone marrow (BM) precursors and leukaemic blasts was compared, it was shown that AML M2 cases with t(8;21) not only had the highest percentages of CD34+ blasts, but in >80% of CD34+ cases the individual blasts expressed higher than normal levels of CD34 antigen (>60 x 103 ABC per cell). In addition, in 73% of this group CD34 antigen was overexpressed in an asynchronous combination with cytoplasmic myeloperoxidase (MPO). Other signs of asynchrony included high CD34 expression with CD15 and/or CD56, as well as aberrant combinations of CD13 with terminal deoxynucleotidyl transferase (TdT) and CD19. These findings demonstrate that asynchrony is identifiable in virtually every case of AML with t(8;21), although it does not always involve the same antigens. M2 cases with t(8;21), mostly CD34+, had a 100% remission rate and 71% 5-year survival rate; other patients with CD34+ or CD34- AML showed 69% and 84% remission rates and 31% and 38% 5-year survival rates, respectively. Consequently, individual markers such as CD34 should be interpreted in relation to other features such as chromosomal changes. These simple methods, which are well suited to quantify the expression of ligands, are a useful contribution to diagnosis: 60% to 65% of M2 cases with t(8;21) are rapidly identified by CD34 overexpression alone. This aberration, together with the other signs of asynchrony seen at presentation, can be used to search for residual leukemia after therapy.

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appeared to be predictive of poor clinical outcome. However, this finding seems to contradict the fact that AML M2 with t(8;21) is a favorable prognostic category despite CD34 positivity of the blasts.

In this study, a total of 517 patients entered onto Medical Research Council (MRC) AML10 therapeutic trials were studied. We describe the overexpression of CD34 antigen in AML M2 with t(8;21) and reveal the characteristic asynchrony when CD34 is studied in combination with MPO. This asynchrony also coincides with other aberrant phenotypic features, contributing to the differential diagnosis and allowing the immunologic monitoring of patients for the presence of residual disease.

**Patients and Methods**

*Selection of patients.* Immunodiagnostic, cytochemical, and cytogenetic results were available on 517 BM and blood samples from patients with AML aged 17 to 55 years (mean, 47.5y). Clinical follow-up data were available from the adult AML10 trial of the MRC of Great Britain. The hematologic diagnosis followed the guidelines of the French-American-British classification based on morphology and cytochemistry. Routine immunophenotyping results were obtained by immunofluorescence microscopy or flow cytometry as described later. The treatment included four courses of intensive chemotherapy consisting of daunorubicin, cytarabine, and thioguanine + 3 and 3 + 8 + 5; melotrexate, adriamycin, cyclophosphamide, and etoposide; and MidAc (mitoxantrone, daunorubicin, cytosine arabinoside). Patients with a matched sibling were allocated to allogeneic BM transplantation (BMT). Those who lacked a donor were randomized to receive either no further treatment or an autologous BMT as the fifth course. The results of this stratification according to the three choices (ie, chemotherapy and allogeneic or autologous BMT) are available from the UK Trial Office, but are omitted here since they do not influence the study conclusions.

Expression of CD34 antigen was quantified in about a third of the cases with t(8;21) and 42 without t(8;21). Finally, a selected group of 18 cases of AML M2 with t(8;21) were further investigated to analyze the various signs of phenotypic asynchrony with a panel of markers used in two- and three-color immunofluorescence tests.

**Control samples.** Fetal BM was obtained from fetal long-bone samples provided by the MRC Tissue Bank, Royal Postgraduate Medical School, London, under the ethical guidelines and permission of the Ethical Committees of both the sending and receiving institutions. The gestational ages, 8 to 18 weeks, were determined from the crown/rump length. BM cells were flushed from the bone cavity with Hanks balanced salt solution, (Gibco, Uxbridge, UK). Normal BM samples were also received from children with idiopathic thrombocytopenia for diagnosis without evidence of BM pathology. KG1 cell line was grown under standard conditions.

**Immunodiagnosis.** Mononuclear cells were separated on Ficoll-Isoaque density gradient 1.077 g/mL. (Lymphoprep; Nycomed, Oslo, Norway). The stored cells were rapidly thawed by diluting in Hanks balanced salt solution at 20°C and washed. Cell suspensions (2.5 to 5 x 10^6 cells per 50 μL) were incubated for 10 to 15 minutes at 20°C with MAbs conjugated with fluorescein isothiocyanate (FITC), green, fluorescein 1: (F11), phycocerythrin (PE), orange, F12), or Peridinin chlorophyll A (red, F13) used as premixed cocktails at pretitrated optimal conditions (Table 1). After washing in phosphate-buffered saline, the stained cells were fixed in 0.5% paraformaldehyde. Isotype-matched FITC- and PE-conjugated murine IgGs were used as negative controls. Five-parameter flow cytometry, ie, three-color IF with forward (FSC) and side (SSC) scatter, was performed on a FACSscan with Paint-a-gatel and Lysys-II softwares (Becton Dickinson, Oxford, UK). List mode files were also transferred to IBM-compatible computer by HP-Reader and analyzed with Winlist software (Verity Software House, Topsham, MI). The percentage of positivity for various MAbs was determined after stratification according to the three choices (ie, chemotherapy and allogeneic or autologous BMT) are available from the UK Trial Office, but are omitted here since they do not influence the study conclusions.

Detection of cytoplasmic and nuclear antigens. During routine immunodiagnosis, detection of cytoplasmic MPO and nuclear TdT was performed on cytopspins using anti-MPO MAB (Dakopatts Ltd, High Wycombe, Bucks, UK) with alkaline phosphatase anti-alkaline phosphatase (Dakopatts), and rabbit anti-TdT serum with goat anti-rabbit Ig labeled with rhodamine (TRITC) as a second layer (Supertech, Bethesda, MD). The percentage of positivity for various MAbs was determined after gating blast populations by their FSC/SSC features. Cases were regarded as CD34+ if more than 20% of cells in the blast gate expressed this antigen.

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Results are given as percentages of positive cells within the blast cell population analyzed by flow cytometry or the APAAP immunoenzyme method.

**Abbreviation: NT, not tested.**

* MABs were directly conjugated as CD13-FITC (WM47; from Dr K. Bradstock, Westmead Hospital, Sydney, Australia), CD15-FITC (CD1-3; Dakopatts), HLA-DR-perCP (RFD2-2; RFHSM), CD14-PE (UCH1; from Professor P. Beverley, ICRF, London, UK), CD34-FITC (BI-C83; Serlab, Crowley, UK), CD34-PE (G1BEND; Professor M.F. Graves, LRF Labs, London, UK), CD56-PE (Leu19; Becton Dickinson, UK), anti-MPO (Dakopatts), and H-TdT-FITC (Supertech, Bethesda, MD) and used on two- and three-color combinations.

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plasmic CD22 (RF84; Royal Free Hospital), CD79 (mb-1; kindly provided by Dr D.Y. Mason, John Radcliffe Hospital, Oxford, UK; and Dakopatts), and cytoplasmic CD3 (Leu 4-PerCP, BD) were investigated by the same methods.

Quantitation of CD34 expression. FACScan was set on logarithmic scale. Instrument settings and channel compensations were controlled by calibrated fluorescence reference beads Quantum (Flow Cytometry Standard, Research Triangle Park, NC). Intensity of CD34 expression was measured as ABC per cell on standard cells with indirect IF using the QIFI kit (Biocytex, Marseille, France) as described previously. Both the first layer, CD34 MAb (B13C5; Seralab Ltd, Crowley Down, UK), and the affinity-purified second layer, goat anti-mouse Ig (Gemi-M FITC; Southern Biotechnology Associates, Birmingham, AL), were used at pretitrated saturated conditions. One standard was CD34+ AML blasts taken from a single patient at the start of the study (P1-AML), frozen in small aliquots, and thawed with more than 90% viability whenever needed throughout the investigations. These P1-AML blasts expressed 64 × 10^3 CD34 mean ABC per cell when tested with the QIFI test. The other standard was a subclone of the KG1 cell line expressing 410 × 10^3 CD34 ABC per cell, a value at the upper limit of the measured range. At the same time, QIFI tests were also performed on CD4+ normal blood lymphocytes with the same Gemi-M FITC second layer, producing 47 to 57 × 10^3 CD4 ABC. These three values together represented the framework to render the quantitative IF tests comparable through the span of studies. At the next stage, directly conjugated CD34 MAb, CD34-FITC (BI-3C5; Seralab) and CD34-PE (QBend, see above), were used at saturating level in a final concentration of 5 μL/100 μL on the same cellular standards (P1-AML and KG1). The bead standards used for direct IF (Quantum Simply Cellular; QSC) from Flow Cytometry Standards (Research Triangle Park, NC) were recalibrated to match the known values of 64 × 10^3 and 410 × 10^3 per cell on P1-AML and KG1, respectively. This was a necessary step to maintain reproducibility between different directly conjugated CD34 reagents. Values for ABC were determined on Winlist with the QuickCal method following linearization of the mean fluorescence intensity. Thus, CD34-PE provided higher MFI values than CD34-FITC, but similar ABC per cell. These two reagents showed an excellent correlation on the various cases of AML (R^2 = 99; not shown) and could therefore be interchangeably used in two- and three-color IF combinations.

Statistics. Life-table analyses with Kaplan-Meier curves were prepared at the MRC Clinical Trials Service Unit, Cancer Studies Unit, Oxford, UK, using the Statistical Analysis System (Cary, NC) and the Mantel-Haenszel chi-square test. The nonparametric Mann-Whitney U test was also applied.

RESULTS

Quantitation of CD34 antigen density in normal BM. The mean percentage of CD34+ cells in the mononuclear fraction of fetal and normal BM was 6.2% (range, 1% to 20%; n = 6). There was a difference in CD34 binding capacity of small- to intermediate-size cells (mean, 26.5 × 10^5 ABC per cell) versus larger cells (mean, 42 × 10^5 ABC per cell), as determined by gating cells of different sizes on the scatter plot and calculating their CD34 ABC values. This was confirmed by double staining with CD10 MAb; CD34+/CD10- cells (B-cell precursors) displayed lower CD34 ABC (mean, 29.5 × 10^5 ABC per cell) than CD34+/CD10- cells with larger size that include early myeloid precursors (mean, 44 × 10^5 ABC per cell). Very few, if any, cells could be observed with more than 60 × 10^5 CD34 ABC per cell; this value can be considered the upper limit of CD34 expression on precursor cells in fetal and normal BM. Double staining for CD34 and MPO in normal and fetal BM showed that among CD34- cells, the MPO+ majority population (R2 gate in Fig 1b) expressed higher levels of CD34 (median, 25 × 10^5 CD34 ABC per cell; Fig 1c) than the rare MPO+ forms (R3 gate in Fig 1b; median, 16 × 10^5 CD34 ABC per cell; Fig 1d).

Incidence of CD34 positivity in AML. When grouped by French-American-British criteria, among 517 cases of AML tested, 103 (19.9%) were M0/M1, 152 (29.4%) M2, and 62 (12.6%) M3, and 174 (33.8%) showed signs of monocytic differentiation, including 121 cases of M4 and 53 of M5 (Fig 2), with 21 cases (4.1%) in other minor categories (not shown). It was found that CD34 positivity, identified by more than 20% CD34+ cells within the blast population, was less frequent in AML M3 and M5 (18% and 28% of cases, respectively) than in the other AML subtypes (39% to 42% of cases); nevertheless, these differences were not statistically significant (Fig 2).

The incidence of CD34 expression was checked among M2 cases with t(8;21). In this group, higher CD34 expression was seen than in the other cases with M2 morphology without t(8;21) (P < .001). In 32 of 38 (t;8;21) cases tested (84.2%) more than 20% leukemic blasts were CD34+, with a mean of 68.5% (range, 20% to 98%). Nevertheless, six cases of M2 with t(8;21) representing 15.8% of t(8;21) cases were CD34+.

Quantitation of CD34 antigen expression in AML. Since both microscopic and flow-cytometric observations indicated that in a number of AML M2 cases CD34 staining was particularly strong, the CD34 ABC was quantified in about one third of all CD34+ leukemias entered into the trial. These were morphologically characterized as seven M0, 18 M1, eight M2 without t(8;21), l1 M2 with t(8;21), and nine with monocytic differentiation (M4/5). CD34 ABC was significantly higher in AML with t(8;21) than in any other group of AML (Fig 3); six of 11 cases displayed values more than twofold higher (300 to >400 × 10^5 ABC per cell) than the highest CD34 ABC (144 × 10^5 ABC per cell) found in other cases of AML without t(8;21). In nine of 11 cases (82%), CD34 ABC was higher than the upper limit of the CD34 range for normal BM (60 × 10^5 ABC per cell).

CD34 ABC was also higher than the mean CD34 expression seen in normal BM (30 × 10^5 ABC) in seven of 18 (38%) M1, three of eight (37.5%) M2 without t(8;21), and three of nine (33%) AML. Nevertheless, only seven of 42 cases (16.7%) of AML without t(8;21) had higher than the maximal levels of CD34 expression seen in normal fetal BM (Fig 3).

Aberrant features of myeloblasts in AML M2 with t(8;21). A total of 18 cases were investigated in further detail, all of which showed t(8;21)-associated cytologic and cytochemical features such as M2 morphology displaying varying degrees of granulocytic maturation, with Auer rods seen in all cases. There was no evidence of any monocytic component on either morphology or esterase staining or any demonstrable erythroid or megakaryocytic abnormalities. The blasts also displayed the typical nuclear indentation with a prominent Golgi region. Granulocyte maturation was abnor-
Fig 1. Quantitative assessment of CD34 expression in normal infant BM (a to d) and in AML M2 with t(8;21) (e to h). Values in the gate R2 in normal BM (b) correspond to the mean values of $25 \times 10^3$ ABC per cell and include both intermediate and large cells. Only some of these cells are MPO-positive ($16 \times 10^3$ CD34 ABC per cell). As a marked contrast, MPO-positive cells (gated in R5) in AML with t(8;21) were as high as $165 \times 10^3$ CD34 ABC per cell, above the normal range. R5 in f can be used as a live gate to search for residual aberrant myeloblasts in patients during therapy.

Fig 2. Incidence of CD34* leukemias among cases of AML. In this study of 494 patients, cells with $>5 \times 10^3$ CD34 ABC per cell were regarded as positive, and cases with $>20\%$ CD34* cells within the blast gate were scored as positive. The numbers shown are percentage positive cases within each French-American-British category.

Fig 3. Quantitation of ABC per cell in 53 cases of CD34* leukemia. Mean ABC values in blast cell populations are shown following quantitation with CD34-FITC calibrated on the standard AML and KG1 cell line. The highest expression is observed in M2 with t(8;21). In six cases, values are $>250 \times 10^3$ ABC per cell.
CD34+ cells seen in normal and fetal BM in nine of 11 cases (81%; Fig 3). Furthermore, in eight of these samples (73%), CD34 showed an asynchronous combination with MPO above the levels seen in normal BM28 (Fig 1). A distinctive live gating (R5 in Figs 1f and h) clearly identified large proportions of malignant blasts, indicating which parameters should primarily be used when remission samples are analyzed from the same patient. The high levels of CD34 expression in combination with another marker of granulocytic differentiation, CD15, were also recorded in seven of 18 cases analyzed (39%), although the double-stained blasts were relatively few in two cases (Table 1).

Finally, strong CD19 expression by CD34+ myeloblasts was detected in three cases, whereas in other samples the CD19+ peak, when studied with the RFB9 MAb used, showed weak positivity but could not be fully separated from negative control; the CD19 ABC per cell was low (not shown). Cytoplasmic CD22 and CD79a, bona fide B-lineage-specific antigens, were negative in all cases, including those that were TdT-positive. Taken together, these observations demonstrate that aberrant and/or asynchronous combinations are identifiable in virtually every case of AML M2 with t(8;21), although these changes are not fully uniform (Fig 4) and in 15% to 20% of AML M2 with t(8;21) do not express strong CD34 positivity (Figs 2 and 3).

### Correlation with clinical outcome
Clinical outcome for 38 patients with t(8;21) and 479 patients with other forms of AML is shown in Table 2 and Fig 5. All patients with t(8;21) obtained complete remissions (CRs) 100%; in this group, significantly fewer relapses and deaths were seen (71% 5-year survival) than among patients who had no t(8;21) (34% survival, P < .0005; Table 2). Taken overall, the CR rate was significantly better in CD34+ patients than in CD34- patients (84% v 75%, P = .008), but there was no survival difference (38% v 37% at 5 years). When the t(8;21) group was studied separately, six CD34+ patients were still all in remission, and 32 CD34- patients had an excellent 62% 5-year survival rate (Fig 5); nevertheless, this CD34+ versus CD34- comparison, due to the small sample size of CD34+ patients with t(8;21), is uninterpretable. When the non-t(8;21) group was analyzed separately, CD34+ patients had a significantly higher CR rate (84%) and marginally better survival (36%) than the CD34- group (69% CR and 31% survival). However, the difference between CD34+ and CD34- subgroups is relatively small compared with the greater impact of cytogenetics.

### DISCUSSION
The AML M2-associated t(8;21)(q22;q22.3) results in fusion of the AML1 gene on chromosome 21 with a gene on chromosome 8 referred to as ETO.22,23 These molecular findings already provide insights into the altered function of the AML1/ETO protein in the processes normally regulated by AML1 gene during myeloid differentiation.1 In this report, we characterize the complexity of these changes when measured in these malignant AML blasts with quantitative immunologic techniques. In all of these cases, irregular control of cellular differentiation can be demonstrated with simple combination staining for precursor cell features such as CD34 or TdT versus signs of myeloid differentiation such as MPO, CD13, and CD15 display. Furthermore, many of these alterations appear to be characteristic for this particular disorder. Further extended use of the quantitative techniques used in this study on a larger cohort of patients will be required to see whether such changes occur at all in AML without t(8;21). The most obvious of these changes is the overexpression of CD34 antigen in MPO-positive malignant myeloblasts. Nevertheless, these changes are not fully uniform (Fig 4), and 16% of AML with t(8;21) are CD34-. Thus, the complexity of the changes argues for a disturbed regulatory control rather than for mere involvement of genes that directly regulate these particular proteins.

One of the main features of our study has been the quantitation of CD34 antigen on normal and leukemic cells in combination staining with other MAbs, eg, for cytoplasmic MPO. This necessitated the use of CD34-FITC and CD34-
PE reagents. If a single reagent is used in an investigation, the molecules equivalent to soluble fluorochromes (MESF units) are adequate to obtain comparative data. In our case, it was more appropriate to introduce standard CD34+ cells that expressed known values for ABC per cell established by the QIFI test in indirect IF28,29,34 to secure the reproducibility of the performance of directly conjugated CD34 used in diagnostic staining combinations (see the methods). In this way, quantitative IF becomes a practical possibility. The same methods have already been applied to document the overexpression of CD10 antigen on 44% of cases with acute lymphoblastic leukemia.

Expression of CD34 in AML has previously been associated with the poorly differentiated French-American-British subgroups M0 and M1,35,36 and several studies have suggested that CD34 positivity may be related to a poor clinical response in terms of CR and/or survival.15,19,22 In this context, the observations of Lanza et al,37 who also find that the "bright" CD34 expression is associated with poor prognosis, are particularly interesting. This is because cytogentic analysis reveals that their AML M2 group lacks cases with t(8;21) but primarily shows -5 and 5q- involvement, a known poor prognostic sign.38 Thus, our investigations, based on large numbers of patients who were uniformly treated in the AML10 multicenter trial,39 may resolve the apparent discrepancies seen in the literature that appear to be due to different proportions of patients with various chromosomal abnormalities in the individual studies. On one hand, when we excluded t(8;21) patients from the evaluation, CD34+ AML cases were less likely to obtain CR and showed slightly inferior 5-year survival as compared with the CD34- group (Fig 5). These findings are reminiscent of the poor prognostic significance of CD34 expression, save the t(8;21) group. On the other hand, we confirm the survival advantage of patients with t(8;21) despite the obviously bright CD34 expression on most of these cases.1,23 In conclusion, these findings indicate that individual markers such as CD34 positivity should not be taken out of the context of the overall clinical and cytogentic presentation, but should be used for patient assessment as part of the diagnosis. Our studies also confirm other observations that emphasize the relevance of chromosomal changes in interpreting the prognostic significance of lymphoid marker expression such as CD19 and CD21,39 as well as CD7,40 in the various forms of AML.

A clear example of the diagnostic advance inherent in our investigation is the use of quantitative IF for rapid diagnosis, as well as remission assessment, in t(8;21) AML. Sensitive polymerase chain reaction techniques have uniformly revealed the persistence of AML1-ETO fusion gene in patients during remission, sometimes as long as 52 months after therapy.41-44 This is a clear step toward a better understanding of a multistep process in malignancy, but it questions the clinical utility of these molecular probes during the remission phase. Residual-disease assessment with immunologic methods in these diseases should now include analysis of CD34+ cells as well as remission assessment, in t(8;21) AML. Sensitive polymerase chain reaction techniques have uniformly revealed the persistence of AML1-ETO fusion gene in patients during remission, sometimes as long as 52 months after therapy.41-44 This is a clear step toward a better understanding of a multistep process in malignancy, but it questions the clinical utility of these molecular probes during the remission phase. Residual-disease assessment with immunologic methods in these diseases should now include analysis of CD34+ cells as well as remission assessment, in t(8;21) AML. Sensitive polymerase chain reaction techniques have uniformly revealed the persistence of AML1-ETO fusion gene in patients during remission, sometimes as long as 52 months after therapy.41-44 This is a clear step toward a better understanding of a multistep process in malignancy, but it questions the clinical utility of these molecular probes during the remission phase. Residual-disease assessment with immunologic methods in these diseases should now include analysis of CD34+ cells as well as remission assessment, in t(8;21) AML. Sensitive polymerase chain reaction techniques have uniformly revealed the persistence of AML1-ETO fusion gene in patients during remission, sometimes as long as 52 months after therapy.41-44 This is a clear step toward a better understanding of a multistep process in malignancy, but it questions the clinical utility of these molecular probes during the remission phase. Residual-disease assessment with immunologic methods in these diseases should now include analysis of CD34+ cells as well as remission assessment, in t(8;21) AML. Sensitive polymerase chain reaction techniques have uniformly revealed the persistence of AML1-ETO fusion gene in patients during remission, sometimes as long as 52 months after therapy.41-44 This is a clear step toward a better understanding of a multistep process in malignancy, but it questions the clinical utility of these molecular probes during the remission phase. Residual-disease assessment with immunologic methods in these diseases should now include analysis of CD34+ cells as well as remission assessment, in t(8;21) AML. Sensitive polymerase chain reaction techniques have uniformly revealed the persistence of AML1-ETO fusion gene in patients during remission, sometimes as long as 52 months after therapy.41-44 This is a clear step toward a better understanding of a multistep process in malignancy, but it questions the clinical utility of these molecular probes during the remission phase.

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