Brief Communication

Residual Disease in AML, a Target that can Move in more than one Direction

The review article "Tumor heterogeneity makes AML a 'moving target' for detection of residual disease" addresses an important issue using leukemia associated immunophenotype (LAIP) for monitoring response to chemotherapy (1). The authors focus on why some AML patients relapse when no residual disease is detected after induction therapy. The LAIP approach to the detection of residual disease presupposes that the phenotype identified at diagnosis remains constant after therapy. In a similar manner, the constancy of genetic abnormalities as a means to detect residual disease using molecular techniques also assumes there is no genetic drift. The authors review the literature demonstrating that changes in both phenotype and genotype are common and may affect the detection of residual disease. The requirement for a diagnostic specimen to establish the LAIP and the frequent shift of phenotype suggests that this approach must be used with great caution when evaluating response to therapy. Although, the authors discuss both the loss and gain of antigens, in the context of LAIP, it is the loss of marker antigens that will affect the accuracy of analysis. Gains of antigens would not be detected in the LAIP analysis which is based on the diagnostic specimen.

A model of clonal evolution is proposed in this review as a potential theoretical basis for this phenotypic/genotypic instability. Clonal evolution, regression or both can cause phenotypic changes that might result in relapse of a clone that could not be detected in the diagnostic specimen or after induction therapy. Alternatively, as the authors propose, a dominant phenotype may obscure other cell populations which become apparent only after induction therapy.

Like acute lymphoblastic leukemia, AML cells are not only phenotypically different from their normal counterparts, each leukemia is unique (2,3). AML differs from other hematopoietic neoplasms with respect to the increased phenotypic heterogeneity observed within the leukemic clone in AML (2). Early studies comparing leukemia phenotypes to normal maturational patterns showed significant maturational heterogeneity as well as evidence for commitment to more than a single lineage (2). Changes in maturational patterns of the clone can result in significant changes in phenotype without the requirement for multiple clonal populations. Such heterogeneity is observed in *de novo* AML found in pediatric (2) as well as adult patients (3).

An example of phenotypic heterogeneity is illustrated in Figure 1 where CD34, HLA-DR, CD11b, CD36, CD38, CD56, and CD7 on the AML blasts reveal multiple cell populations in a 3-year old patient at presentation. The standard representation of multiparameter data, most often reported as percent positive cells, does not reveal the complexity of the composition of the leukemia. The leukemia in this patient comprised 40% of the nonerythroid cells in the bone marrow aspirate. The proportion of positive cells in the tumor varied for each antigen tested: CD34 = 33%; HLA-DR = 32%; CD11b = 53%; CD36 = 91%; CD38 = 74%; CD7 = 32%, and CD56 = 43%. By combining these parameters in a logical manner to assess the maturation of myeloid progenitor cells using a multidimensional data analysis approach, the basis of this heterogeneity is better understood, Figure 1. Two clearly identifiable cell populations come into focus when the antigenic relationships and physical characteristics between all parameters are maintained by creating a multidimensional data space. The bright green and yellow population is identified by higher intensity CD45 expression while the dark green and orange population expresses lower intensity CD45 (Fig. 1B). These populations are well separated by other markers including CD11b, HLA-DR, CD38, CD36, CD56, and CD7. Superimposed on these distinct cell clusters is the variation in intensity of CD34. The brightest CD34 cells (yellow and orange) are the least mature of the neoplastic cells while the CD34 negative component of each cell population (dark and bright green) represent a more mature phenotype. After induction chemotherapy any of these populations might be detected or missed depending on the LAIP selected to monitor the patient. A shift in phenotype may result from the original clone not maturing in exactly the same manner after chemotherapy or could be caused by the expansion of a minor, refractory clone not apparent in the diagnostic specimen.

The combination of maturation as well as a hidden clone is illustrated in Figure 2 for a 13-year old patient with AML demonstrating t(11;19) cytogenetic abnormality. At presentation, the abnormal cells included not only the progenitor cells (CD34 positive, 12%, CD117 positive, 7.4%) but also demonstrated abnormal maturation to both the monocytes (light blue) and the neutrophils (dark blue), Figures 2A–2D. Following induction chemotherapy, the maturing monocytes and neutrophils were normal phenotype, Figures 2E–2G, however, an abnormal, CD19 positive myeloid progenitor cell was identified at 0.5% that was not detected in the diagnostic specimen (Figs. 2D and 2H). This abnormal phenotype was identified in all subsequent specimens following a second induction, and two rounds of intensification therapy. As this phenotype was not detected in the diagnostic specimen, and its similarity to regenerating B lymphoblasts, this CD19 positive cell population was sorted and shown to be positive for MLL rearrangement by fluorescence *in situ* hybridization in 95% of the purified cells. These results confirm, by an independent method, that this aberrant myeloid progenitor cell population represents residual leukemia.

AML in the adult population is further confounded by AML arising from MDS where the abnormal progenitor cells (20–

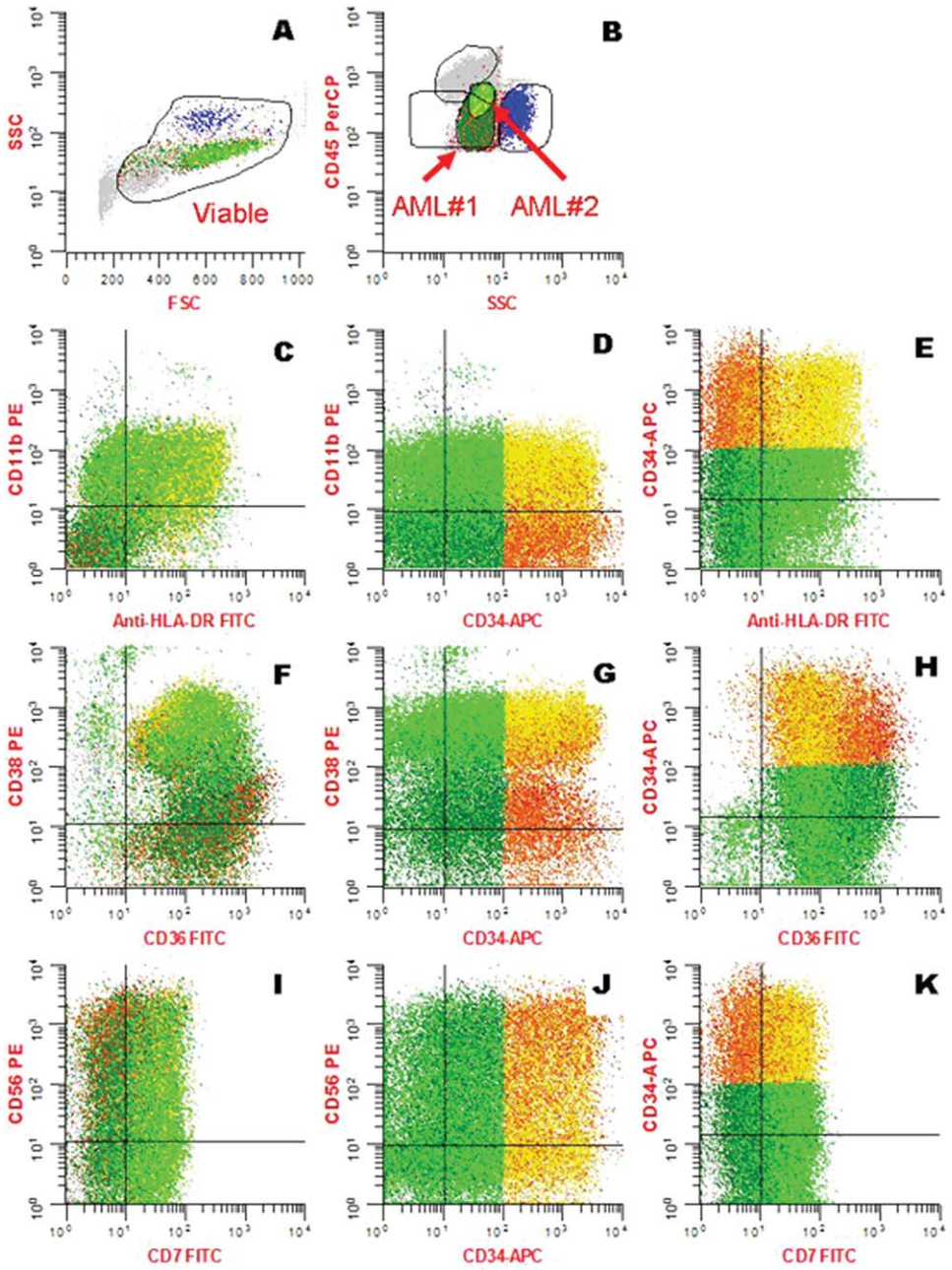

FIG. 1. Multidimensional analysis of leukemia combines all parameters maintaining the spacial relationships for all characteristics. Two populations of cells were identified in this leukemia identified in a 3-year old patient based on CD45 and SSC (**B**). AML#1 was colored dark green or orange (depending on expression of CD34) while AML#2 was colored bright green and yellow. The two groups of cells, presumed to be two clones, are readily apparent when the other combinations of antigens are displayed (**C–K**). These two clones both demonstrate maturational heterogeneity identified by the loss of CD34 expression, orange to dark green and yellow to bright green. Interestingly, CD7 is expressed on AML#2 while CD56 is predominantly expressed on AML#1. The cell population identified as blue contains all maturing myeloid cells while the gray population is comprised of mature lymphocytes. The total number of displayed events in (**A**) is lower than in the other plots to better visualize the size of the abnormal cells. The procedure for staining the bone marrow cells, flow cytometer, source of antibodies, and data analysis are described in Ref. (4).

30%) may be a minority of the abnormal maturing myeloid cells as demonstrated in the previous case. In AML with 20% blasts, the majority of the aberrant cells may not be phenotypic progenitor cells (i.e., positive for CD34 or CD117) but may be comprised of myeloid cells extending from promyelocytes all the way to mature neutrophils and/or monocytes. Following chemotherapy, the cells with an immature phenotype may be eliminated leaving the abnormal maturing myeloid cells. This scenario places the patient back into a myelodysplastic state, a more extreme form of clonal regression.

A "difference from normal" approach using multidimensional flow cytometry allows for a comprehensive analysis of the heterogeneous cell populations that can be observed in acute myeloid

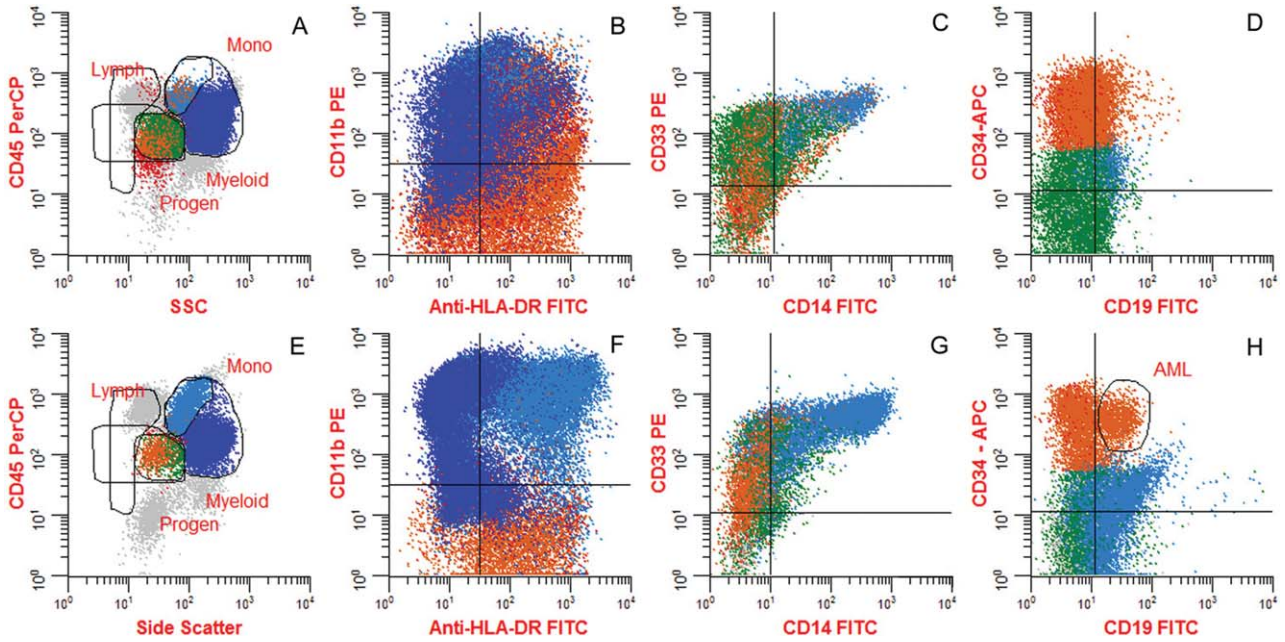

FIG. 2. An AML specimen with t(II;19) from a 13-year old patient exhibited low proportions of abnormal progenitor cells (CD34 +, orange) as well as abnormal maturing monocytes (light blue) and neutrophils (dark blue) at diagnosis (**A–D**). After the first cycle of induction chemotherapy, the phenotypes of the monocytes and neutrophils normalized (**E–G**), however, an abnormal myeloid progenitor cell population expressing CD19 was detected at 0.5% (**H**). The CD19 positive population was sorted for fluorescence *in situ* hybridization studies revealing 95% harboring a rearranged MLL gene. Panel B was gated on monocytes, neutrophils, and progenitor cells while panels C and D were gated on progenitor cells and monocytes. Gating of panels E–H was identical to the corresponding upper panels.

leukemia (4). Not only can all cell populations, abnormal or otherwise, be defined; the very nature of this approach, when applied correctly, is immune from the effects of tumor heterogeneity, phenotypic drift and clonal selection. Knowledge of the diagnostic phenotype is not required for detection of residual disease (minimal or otherwise) but should be used whenever possible, as this knowledge enhances both the sensitivity and the specificity of the assay.

The authors do not fully address another reason for relapse in patients with no detectable residual disease. The residual disease simply may be below the detection limit of the assay, 10^{-2}–10^{-4}, or may not even be in the bone marrow but residing in an extramedullary site. The lower level of detection of residual disease analysis is a key factor in defining the assay but in the articles cited, no consistent level of detection was identified. If the leukemia is below detection limit, flow cytometry can be used to closely monitor patients to detect early relapse followed by intervention. This approach may be a more effective way to individualize treatment.

The focus of this review, asking why do some patients who do not have detectable residual disease eventually relapse, skirts a more important clinical issue which is the reciprocal problem, that is, are all of the patients defined as positive for residual disease really harboring leukemia? Using the LAIP approach, each reagent combination is unique to that patient. The separation between the leukemia and a stressed bone marrow may be less than sufficient to distinguish between normal and abnormal cells at the defined cutoff. Therefore, normal regenerating bone marrow cells may be included within the LAIP window resulting in the classification of the patient as having leukemia when, in fact, they are normal. The classification of patients as poor risk using residual disease detection must be completely specific (in this context: no false positive cases) to avoid over treating a group of otherwise intermediate or good risk patients. Specificity in identifying only true positive cases is more important than pushing down the limit of detection. Therefore, a review of minimal residual disease assays used to identify risk categories should also consider how accurately the positive group is defined. This is a more difficult parameter to access as the inclusion of good risk patients in the poor risk group, especially at the lower levels of detection, improves the overall survival of that patient cohort.

LITERATURE CITED

1. Zeijlemaker W, Gratama J, Schuurhuis G. Tumor heterogeneity makes AML a "moving target" for detection of residual disease. Cytometry B Clin Cytom (in press) DOI: 10.1002/cyto.b.21134.
2. Terstappen LWMM, Loken MR. Myeloid cell differentiation in normal bone marrow and acute myeloid leukemia assessed by multi-dimensional flow cytometry. Anal Cell Path 1990;2:229–240.
3. Terstappen LWMM, Konemann S, Safford M, Loken MR, Zurlutter K, Buchner TH, Hiddemann W, Wormann B. Flow cytometric characterization of Acute Myeloid Leukemia. Part II. Phenotypic heterogeneity at diagnosis. Leukemia 1991;6:70–80.
4. Loken, MR, Alonzo TA, Pardo L, Gerbing RB, Raimondi SC, Hirsch BA, Ho PA, Franklin J, Cooper TM, Gamis AS, Meshinchi S. Residual disease detected by multidimensional flow cytometry signifies high relapse risk in patients with *de novo* acute myeloid leukemia: a report from Children's Oncology Group. Blood 2012;120:1581–1588.

Michael R. Loken
Hematologics, Inc. 3161
Elliot Avenue Seattle, WA 98121
E-mail: mrloken@hematologics.com

Published online in Wiley Online Library (wileyonlinelibrary.com).
DOI: 10.1002/cyto.b.21140