Consistent Quantitative Gene Product Expression: #3. Invariance with Age

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Abstract
The quantitative expression of cell surface antigens and light scattering properties of five cellular reference populations in stressed bone marrow specimens were compared between pediatric and adult patients treated for acute myeloid leukemia (AML). The mean intensity of each antigen as well as the within patient and between patient variability showed striking consistency between the two different age groups. The only difference between the groups of specimens was the proportion of progenitor cells in the adult cohort averaged less than three times the proportion in the pediatric cohort. These data show that the amounts of gene products expressed on bone marrow cells are invariant with age.

Key terms
flow cytometry; bone marrow aspirates; adult; quantitative antigen expression; support vector machines

INTRODUCTION
A companion manuscript identified five key reference populations that could be routinely detected in regenerating bone marrow aspirate specimens from pediatric patients treated on a single clinical protocol early post chemotherapy for acute myeloid leukemia (AML) (1). The data demonstrated that the amounts of gene products were highly regulated, with many gene products exhibiting minimal variability within individuals and even less variability between individuals. In this manuscript we extend this analysis to adults recovering from chemotherapy for AML.

MATERIALS AND METHODS
Patient Data Set
A total of 50 randomly selected, adult acute myeloid leukemia patients (age 23–73, median = 55.5) obtained post chemotherapy were identified as having no evidence of residual disease (2). Patients eligible for this study satisfied three criteria: (1) a prior history of treatment for AML, (2) submission of a normal, regenerating bone marrow aspirate without detectable residual disease by flow cytometry, and (3) evidence of high specimen quality with minimal hemodilution (3). In contrast to the pediatric specimens described in the companion manuscript, the bone marrow specimens from the adults were not part of a clinical study but were submitted for monitoring response to therapy in patients with AML. Therefore, the specimens were more heterogeneous not only with respect to age of the individual, but also were not standardized to a single therapy regimen nor to a single time point post therapy. Adult specimens from both pre- and post-hematopoietic stem cell transplant therapy were included in this study. This study was conducted following the guidelines of the Declaration of Helsinki – Ethical Principles for Medical Research Involving Human Subjects.
Specimen Collection
Bone marrow aspirates were collected in heparin (the preferred anti-coagulant) or EDTA. The data were obtained over a period of 2 years and 6 months (overlapping in time with the analysis of the specimens for the pediatric study) using three separate flow cytometers, multiple reagent lots and processed by multiple technicians.

Flow Cytometry
Specimens were processed as routine clinical bone marrow aspirates as previously described (2). Briefly, 100 µL of bone marrow was added to cocktails of pre-tittered antibodies at room temperature in the dark. Red blood cells were lysed using 3.5 mL of buffered NH₄Cl (0.83%) at 37°C for 5 min before centrifugation at 300G. Cells were then washed with 3 mL of phosphate buffered saline containing 2% fetal calf serum and re-suspended to 0.5 mL in 1% paraformaldehyde for analysis on one of three FACS Calibur instruments (Becton Dickinson Biosciences, San Jose, CA). 200,000 events were collected for each tube. The flow cytometers were cross standardized and calibrated using RCP-30A and RFP-30A beads (Spherotech, Lake Forest, IL) with spectral compensation performed using peripheral blood cells labeled with CD4 (SK3, BD) conjugated to fluorescein (FITC), phycoerythrin (PE),...
peridinin chlorophyll protein (PerCP) or allophycocyanin (APC). Eight combinations of antibodies were used as previously described (1,4).

Support Vector Machines

Support vector machines (SVMs) were trained on 27 pediatric bone marrow specimens from patients recovering from chemotherapy (1,4). These same SVMs were used to analyze the adult patients. The time period for acquisition of these data overlapped that of the pediatric group with data collected over greater than a 2-year period. A more detailed description of the analytic approach is contained in a companion article (4).

RESULTS

The same five reference populations could be identified in all adult specimens as in the pediatric study: (1) Mature lymphocytes, identified by high CD45 and low log SSC, were detected in all eight reagent tubes, (2) Uncommitted progenitor cells, identified by high expression of CD34 and intermediate CD33, were detected in all eight reagent combinations, (3) Promyelocytes, identified by high log SSC without expression of HLA-DR or CD11b, (4) Mature monocytes, identified by high expression of CD33 and CD14, and (5) Mature neutrophils, identified by high expression of CD16 and CD13.

The parameter means and variation characteristics of CD45 and log SSC were determined for all five adult populations and compared with the results from the pediatric study (Table 1). The CD45 and log SSC parameter means for all five reference populations were indistinguishable between the pediatric and adult patients. Likewise, the CD45 and log SSC replicate variation characteristics were very similar between the pediatric and adult populations. Notably, the within-patient, between-patient, and replicate variation were slightly elevated for the uncommitted progenitor cells in the adult group. This small increase could only be observed by statistical analysis and could not be detected by visually comparing the populations on a four-decade log scale. The between patient variability, i.e. variability of the means between patients, was always less than the within patient variability for both the adult and pediatric groups for CD45 and log SSC.

CD34 expression on the uncommitted progenitor cells was compared between the pediatric and adult patients (Fig. 1). The parameter means for both the pediatric and the adult were essentially identical (3.14 vs. 3.13 log units) (Table 2). The replicate variability between the eight tubes for CD34 intensity was the same between the pediatric and adult Group (0.032 vs. 0.034 log units) (Table 2). The within patient variation (0.15 vs. 0.15 log units) as well as the between patient variability (0.065 vs. 0.066 log units) was also the same in pediatric and adult patients. The major difference between pediatric and adults was observed in the total number of uncommitted progenitor cells (Fig. 1). The proportion of total events identified as uncommitted progenitor cells in the pediatric group (mean > 3% of total cellular events) was >3 times higher as compared to the proportion of total events identified as uncommitted progenitor cells within the adult group (mean = 0.35% of total cellular events).

A comparison of the gene products expressed on mature monocytes demonstrated that the highly regulated antigens CD14, CD33, and CD64 demonstrated similar mean intensities, within patient variation, and between patient variation for both pediatric and adults (Table 3). CD64 expression is broader in both pediatric and adults and again demonstrated similar means intensities, within patient variation, and between patient variation. In both patient populations, the between patient variation for CD33 was also greater than the within patient variation.

The mature neutrophils, identified by high expression of CD16 and CD13, also demonstrated reproducible results comparing pediatric and adult patient populations (Table 4). The differences could only be identified using statistical analysis and could not be seen in visual inspection on a four-decade (log) scale. As shown for the pediatric group, CD16 demonstrates a tighter within patient variability than CD13 for the adults. Therefore, the position of the mature neutrophils with respect to CD13 and CD16 is the same between pediatric and adult patients.

### Table 2. Intensity of CD34 on uncommitted progenitor cells is the same between pediatric and adult patients

| UNCOMMITTED PROGENITORS | PEDIATRIC CD34 | ADULT CD34 |
|-------------------------|----------------|------------|
| Parameter mean          | 3.14           | 3.13       |
| Between patient variation | 0.065         | 0.066      |
| Within patient variation | 0.15          | 0.15       |
| Replicate variation n = 8 | 0.032          | 0.034      |

### Table 3. Mature monocytes express the same intensity of CD14, CD33, CD36 and CD64 in pediatric and adult patients

| MATURE MONOCYTES | CD14 PEDIATRIC | CD14 ADULT | CD33 PEDIATRIC | CD33 ADULT | CD36 PEDIATRIC | CD36 ADULT | CD64 PEDIATRIC | CD64 ADULT |
|------------------|----------------|------------|----------------|------------|----------------|------------|----------------|------------|
| Parameter mean   | 2.54           | 2.53       | 2.74           | 2.82       | 2.88           | 3.01       | 2.99           | 2.99       |
| Between patient variation | 0.062         | 0.082      | 0.25           | 0.27       | 0.11           | 0.11       | 0.21           | 0.22       |
| Within patient variation | 0.18          | 0.17       | 0.14           | 0.13       | 0.18           | 0.21       | 0.33           | 0.28       |

### Table 4. Mature neutrophils express the same intensity of CD16 and CD13 in pediatric and adult patients

| MATURE NEUTROPHILS | CD16 PED | CD16 ADULT | CD13 PED | CD13 ADULT |
|--------------------|---------|------------|---------|------------|
| Parameter mean     | 3.07    | 3.11       | 2.81    | 2.79       |
| Between patient variation | 0.11   | 0.11       | 0.15    | 0.20       |
| Within patient variation | 0.16   | 0.18       | 0.26    | 0.26       |
DISCUSSION

The data presented in this manuscript extend the study of constancy of gene product expression identified in stressed bone marrow specimens from pediatric to adult patients. The adult patients were not standardized to a single chemotherapeutic regimen nor were they standardized to a specific time after chemotherapy as specified in the pediatric study. In fact, many of the adult patients were assessed following a hematopoietic stem cell transplant.

In normal bone marrow, hematopoiesis is characterized by reproducible changes in the quantitative expression of surface gene products, independent of age or marrow stress. Infant, adolescent, adult, and elderly patients exhibit identical phenotypic patterns on the investigated reference populations, even during the administration of chemotherapy and after bone marrow transplants. Taken together, these data illustrate that the quantitative amount of surface gene product expression is a biologic constant, suggesting that in order for a cell to properly mature, the amounts of surface antigens must be precisely regulated.

Although intensity relationships are identical between individuals, the proportion of cells in various maturational stages may vary. The adult population had a substantially decreased proportion of events identified as uncommitted progenitor cells compared to the pediatric population. These data suggest that the adult population may have a distinct kinetic response to chemotherapy, regenerating progenitor cells at a slower rate than the pediatric population. The decreased number of events in this minor population can increase the variability in calculating the mean and variation characteristics of this small population, and may be responsible for the observed increase in replicate and between patient variability in CD45 and log SSC for the uncommitted progenitor cells.

The data presented in this manuscript demonstrate a consistency in cellular gene product expression as well as cellular characteristics such as log SSC. Although the proportions of different cell populations can vary, the amounts of gene products expressed at specific stages of development are identical between the pediatric and adult group and demonstrate a high level of regulation in these key cellular characteristics. These data indicate that during the maturational process the amounts of gene products expressed are identical between pediatric, adult and even elderly patients. It appears that quantitative gene product expression is an inherent property of maturation of each cell type, independent of age and marrow stress. The mechanism controlling this tight gene product regulation has yet to be elucidated.

LITERATURE CITED

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