Cytometry Part B (Clinical Cytometry) 80B:354–361 (2011)

Original Article

Cell-Cycle Distribution of Different Cell Compartments in Normal Versus Reactive Bone Marrow: A Frame of Reference for the Study of Dysplastic Hematopoiesis

Sergio Matarraz,[1] Carlos Fernandez,[1] Manuel Albors,[2] Cristina Teodosio,[1] Antonio López,[1] María Jara-Acevedo,[1] Carlos Cervero,[3] Gonzalo Caballero,[4] Oliver Gutierrez,[5] and Alberto Orfao[1]*

[1]Servicio de Citometría and Departamento de Medicina, Centro de Investigación del Cáncer, Instituto de Biología Celular y Molecular del Cáncer, CSIC-USAL, Universidad de Salamanca, Salamanca, Spain

[2]Servicio de Hematología, Hospital Juan Canalejo, La Coruña, Spain

[3]Servicio de Hematología, Hospital Virgen de la Luz, Cuenca, Spain

[4]Servicio de Hematología, Hospital San Jorge, Huesca, Spain

[5]Servicio de Hematología, Hospital Rio Hortega, Valladolid, Spain

Limited information is currently available about the proliferation activity and cell-cycle distribution of different bone marrow (BM) cell subsets defined according to their lineage and maturation stage in normal versus cytopenia-associated reactive BM samples. Here, we report a three-color flow cytometry approach to investigate the cell-cycle distribution of different BM cell compartments—$CD34^+$ hematopoietic progenitor and precursor cells (HPC), maturing neutrophils and monocytic cells, mature lymphocytes, eosinophils, and nucleated red blood cell precursors (NRBC)—from normal ($n = 47$) versus cytopenia-associated reactive ($n = 47$) BM samples. Highly similar proliferation profiles were detected in normal versus reactive BM, with a higher proliferation index (PI) for the more immature $CD34^+$ HPC, $CD11b^-$ maturing neutrophils and NRBC versus other BM cell compartments. The only differences observed between normal and reactive BM were restricted to the more mature ($CD13^{hi}/CD11b^+$) bands/neutrophils and to monocytic cells, which showed an increased PI (0.9% ± 0.8% vs. 0.6% ± 0.5% and 6 ± 3.6 vs. 4.6 ± 4.5, respectively) at the expense of a lower PI of $CD34^+$ HPC in reactive conditions. Of note, bands/mature neutrophils and mature lymphocytes showed either residual numbers or absence of $S + G_2/M$-phase cells in both normal and reactive BM. Our results suggest that a slight shift of proliferation from the early precursors to the more mature granulomonocytic compartment occurs in reactive BM, which could reflect an attempt of the hematopoietic system to rapidly produce functional neutrophils and monocytes, at the expense of a lower expansion of the minor compartments of $CD34^+$ HPC.

Key terms: proliferation index; cell cycle; bone marrow; flow cytometry

How to cite this article: Matarraz S, Fernandez C, Albors M, Teodosio C, López A, Jara-Acevedo M, Cervero C, Caballero G, Gutierrez O, Orfao A. Cell-cycle distribution of different cell compartments in normal versus reactive bone marrow: A frame of reference for the study of dysplastic hematopoiesis. Cytometry Part B 2011; 80B: 354–361.

Grant sponsor: Instituto de Salud Carlos III, Ministerio de Sanidad y Consumo, Madrid, Spain; Grant number: RTICC RD06/0020/0035; Grant sponsor: Ministerio de Ciencia e Innovación of Spain; Grant number: PI08/90881; Grant sponsor: Ministerio de Ciencia e Innovación of Spain-Universidad de Salamanca (Programa Nacional Personal Técnico de Apoyo a la Investigación).

*Correspondence to: Alberto Orfao, Servicio General de Citometría, Centro de Investigación del Cáncer, Campus Miguel de Unamuno, 37007-Salamanca, Spain. E-mail: orfao@usal.es.

Received 4 March 2011; Revision 28 April 2011; Accepted 4 May 2011

Published online 2 June 2011 in Wiley Online Library (wileyonlinelibrary.com).
DOI: 10.1002/cyto.b.20605

Cell cycle plays a crucial role in the biology of both prokaryotes and eukaryotes (1–4). In human embrionic stages and early ages after birth, cell proliferation is a key function for the development of adult tissues (5,6); later on, it is crucial in fundamental life processes such as cell differentiation and apoptosis, and other relevant biologic mechanisms related to cell development, tissue maintenance, regeneration, and repair (6–11). Within the hematopoietic system, deregulation of the cell cycle has been associated with bone marrow (BM) failure (12–14) and prognosis of several hematological malignancies such as acute myeloblastic leukemia (AML) and multiple myeloma, among others (15,16). Despite this, a careful analysis of the literature shows that limited information has been reported (17) about the proliferation activity and cell-cycle distribution of different compartments of BM cells defined according to their lineage and maturation stage in normal versus cytopenia-associated reactive BM samples.

Flow cytometry immunophenotyping provides a unique tool to assess the distribution of different lineage- and maturation-associated cell compartments in normal versus reactive BM (18,19). At the same time, the proliferation index (PI) of each cellular compartment may be determined in primary samples using supravital DNA dyes such as DRAQ5, in combination with multiple cell surface stainings (20,21). Although such approaches have been applied to the analysis of the PI of normal versus neoplastic B-cells, (17), to the best of our knowledge, no study has been reported so far in which this has been assessed for other BM cell populations in normal versus cytopenia-associated reactive BM samples.

In the present study, we used a three-color flow cytometry approach to investigate the cell-cycle distribution of different BM cell compartments—$CD34^+$ hematopoietic progenitor and precursor cells (HPC), maturing neutrophils and monocytic cells, mature lymphocytes, eosinophils, and nucleated red blood cell precursors (NRBC)—in normal ($n = 47$) versus cytopenia-associated reactive ($n = 47$) BM samples, as a frame of reference for the evaluation of cell-cycle alterations in dysplastic BM samples from patients with malignant myeloid disorders—for example, myelodysplastic syndromes (MDS), AML, or myeloproliferative neoplasms.

MATERIALS AND METHODS

BM Samples

A total of 94 freshly obtained, EDTA-anticoagulated normal ($n = 47$) and reactive ($n = 47$) BM samples from an identical number of individuals (46 men and 48 women; mean age of 67 years, ranging from 47 to 79 years) were collected at the University Hospital of Salamanca (Spain). Normal BM samples were obtained from healthy volunteer donors and during routine surgical procedures from individuals undergoing orthopaedic surgery—both groups of subjects had normal blood cell counts—while reactive samples corresponded to patients with iron and/or vitamin B_{12}/folic acid deficiency associated with anemia and other toxic (e.g., drug-induced) or reactive (e.g., idiopathic) cytopenias, including infection-associated leukopenias. None of the reactive samples showed clonal hematopoiesis, based on the absence of cytogenetic abnormalities, as assessed by fluorescence in situ hybridization (FISH) and/or a polyclonal pattern of inactivation of chromosome X, as specifically evaluated in females by the human androgen receptor assay (HUMARA) test. All BM samples were obtained after informed consent was given by each subject according to the recommendations of the local Ethics Committee, and samples were systematically studied within the first 18 h after they were obtained.

Cell-Cycle Analysis

Analysis of the distribution of different compartments of BM cells along the G_0/G_1, S, and G_2/M cell cycle phases was performed immediately after samples were obtained using triple-stainings for nuclear DNA and two cell surface antigens. Briefly, EDTA-anticoagulated whole BM samples—cell concentration adjusted with phosphate buffered saline (PBS; pH = 7.4) to 10^6 cells in 100 μl/tube—were incubated in two separate aliquots for 10 min in the dark at room temperature (RT) with saturating amounts (10 μl of each reagent) of the following combinations of fluorescein isothiocyanate-/phycoerythrin-conjugated monoclonal antibodies purchased from Becton Dickinson Biosciences (BDB; San Jose, CA): CD45/CD34, CD11b/CD13. Once the incubation period was completed, 2 ml of FACS lysing solution (BDB) diluted 1/10 (vol/vol) in distilled water was added to each tube and another incubation performed for 5 min in the dark (RT). Afterward, cells were washed with 2 ml of PBS and the cell pellet resuspended in 0.5 ml PBS. Then, 3 μl of DRAQ5™ (Vitro SA, Madrid, Spain) was added to each tube, and another incubation was performed for 10 min in the dark at RT. Immediately after this incubation, sample aliquots were measured in a FACSCanto II™ flow cytometer (BDB) using the FACSDiva software program (BDB). For each sample aliquot, information about $>1 \times 10^5$ cells corresponding to the whole BM cellularity was measured and stored; for each cell population, information on $>1 \times 10^3$ cells was collected for cell-cycle analysis purposes. For data analysis, the INFINICYT™ software program (Cytognos SL, Salamanca, Spain) was used. The overall percentage of proliferating cells, including those cells within the S plus G_2/M cell-cycle phases, was identified as those cells showing a brighter staining of DRAQ5 than those included in the G_0/G_1 peak (Fig. 1A) (17,20).

For each sample, the following cell populations were identified after excluding dead cells and cell doublets in a sideward light scatter (SSC) versus DRAQ5-fluorescence area and a DRAQ5-fluorescence area versus DRAQ5-fluorescence width bivariate dot plot (Fig. 1A): total $CD34^+$ HPC ($CD45^{lo}/CD34^+$ events), (22) $CD34^+$ myeloid HPC ($SSC^{int}/CD34^+$), $CD34^+$ lymphoid HPC ($SSC^{lo}/CD34^+$ cells), nucleated red cell precursors ($SSC^{lo/very\text{-}lo}/CD45^-$ events), monocytic cells ($SSC^{int}/CD45^{int/hi}/CD11b^{hi}/CD13^{hi}$ cells), eosinophils (SSC^{hi}/autofluorescent cells), mature lymphocytes ($CD45^{hi}$/

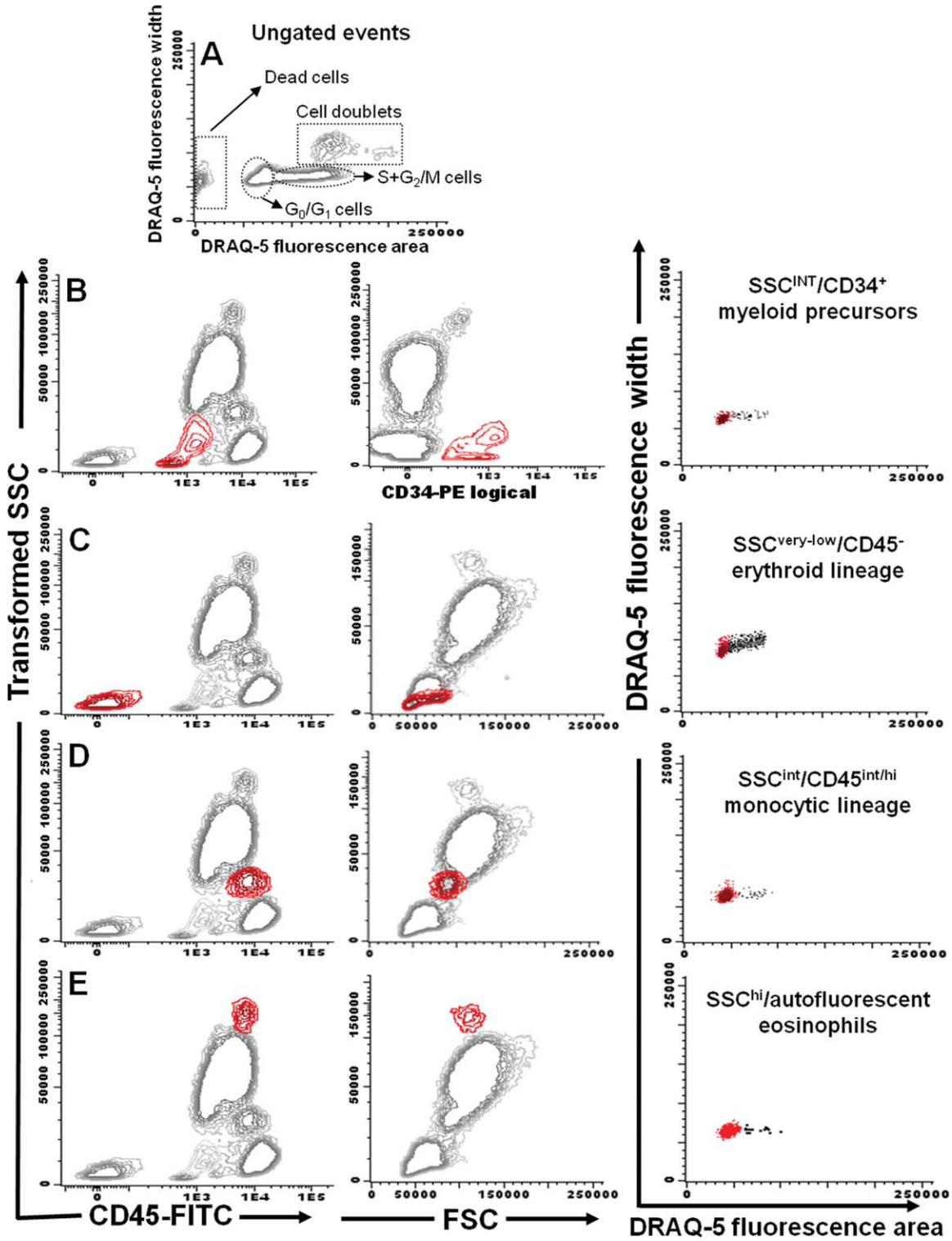

FIG. 1. Representative bivariate dot plots of a normal bone marrow (BM) sample, illustrating the gating strategy used for the immunophenotypic identification of the different populations of BM cells analyzed (excluding neutrophils and mature lymphocytes) (rows B–E) and the analysis of their cell-cycle distribution according to their DNA cell content (right column); $S + G_2/M$ phase cells within each BM cell population are depicted in black. Panel A depicts the strategy used to discard dead cells and doublets. [Color figure can be viewed in the online issue, which is available at wileyonlinelibrary.com.]

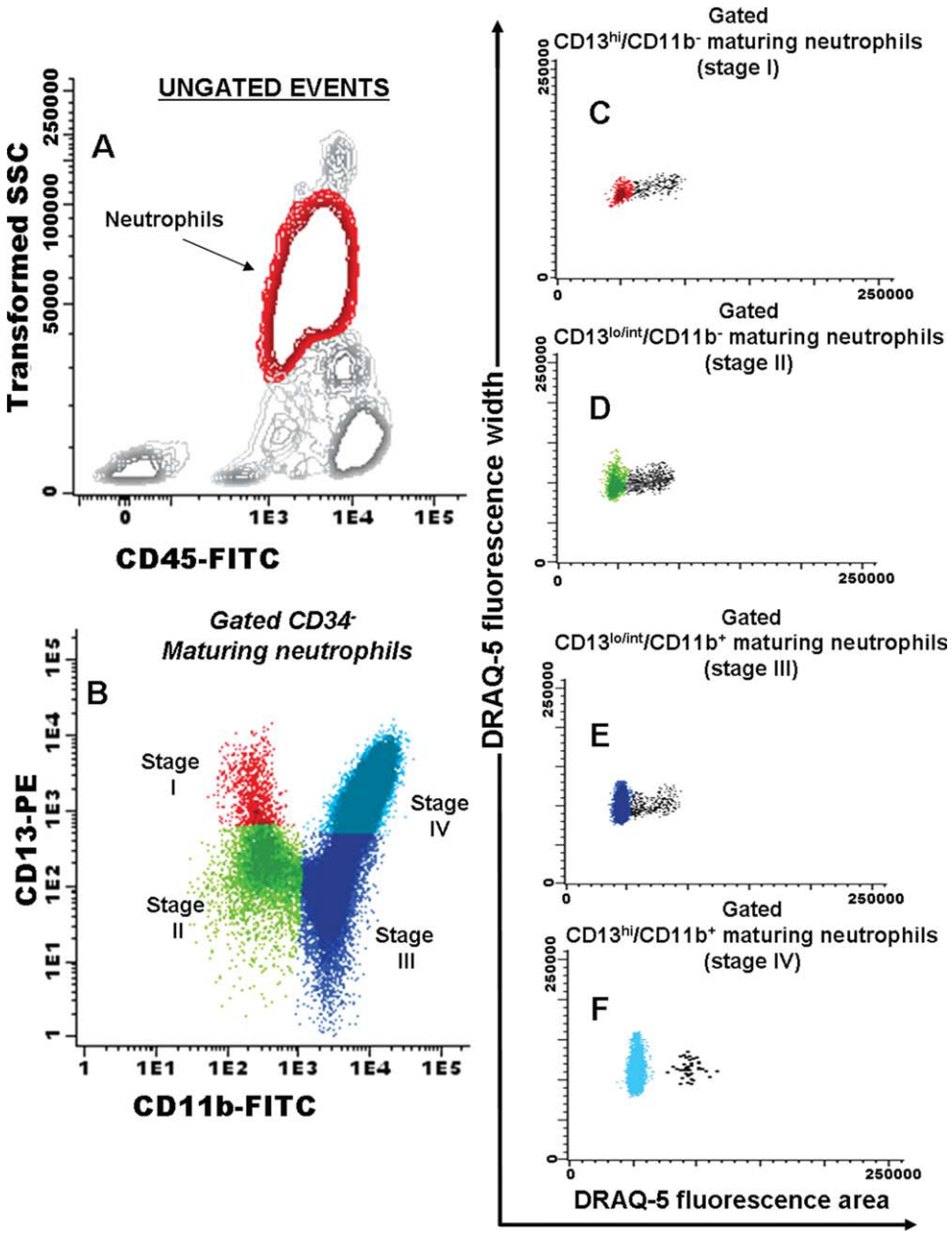

FIG. 2. Bivariate SSC versus CD45 and CD13 versus CD11b dot plot histograms of a normal bone marrow (BM) sample depicting the population of gated $CD34^-$ maturing neutrophils (panel A) and their different maturation stages—stage I, $CD13^{hi}/CD11b^-$; stage II, $CD13^{lo/int}/CD11b^-$; stage III, $CD13^{lo/int}/CD11b^+$, and; stage IV, $CD13^{hi}/CD11b^+$ (panel B). In panels C–F, the analysis of the proliferation index (PI) of the different maturation-associated compartments of BM neutrophils defined in panel B is displayed (black dots correspond to $S + G_2/M$ phase maturing neutrophils).

SSC^{lo}), and maturing neutrophils ($CD45^{lo}/SSC^{int/hi}$); in addition, these later cells (neutrophils) were further subdivided into four maturation-associated cells subsets: $CD13^{hi}/CD11b^-$, $CD13^{lo/int}/CD11b^-$, $CD13^{lo/int}/CD11b^+$, and $CD13^{hi}/CD11b^+$ maturing neutrophils (Fig. 2) (19). The specific immunophenotypic characteristics of mature lymphocytes were taken as a standard to define the relative position of the different compartments of BM precursors in a CD45 versus SSC bivariate dot plot. For each of the above-listed cell populations, the distribution along the G_0/G_1, S, and G_2/M cell-cycle phases was calculated as previously described (Figs. 1B–1E and 2C–2F) (15). Mean coefficient of variation for the G_0/G_1 peak of the different cell populations was of 6.3% ± 1.5%.

Conventional Karyotyping, FISH, and HUMARA Studies

Cytogenetic analysis of BM samples was performed according to standard procedures (23) and interpreted using the International System for Cytogenetic Nomenclature criteria (24). In addition, double-staining interphase

Table 1
Distribution of Different Maturation-Associated Compartments Of Cells (Including Distinct Subsets of Maturing Neutrophil Lineage Cells) in Normal (n = 47) versus Reactive (n = 47) Bone Marrow (BM)

	Distribution of BM cell populations				
	Normal BM ($N = 47$)		Reactive BM ($N = 47$)		
	% of specific cell subsets	% of total BM	% of specific cell subsets	% of total BM	*P*-value
Total $CD34^+$ HPC	–	0.9 ± 0.8 (0.06–4.5)	–	0.7% ± 0.4% (0.2–2%)	NS
$CD34^+$ nonlymphoid precursors	93% ± 8.5% (70–100%)	0.9% ± 0.8% (0.06–4.4)	92% ± 7% (72–100%)	0.6% ± 0.4% (0.2–2%)	NS
$CD34^+$ lymphoid precursors	6.5% ± 8% (0–30%)	0.07% ± 0.1% (0–0.7%)	7.6% ± 7% (0–27.5%)	0.07% ± 0.1% (0–0.7%)	NS
Maturing neutrophils	–	60% ± 9% (44–78%)	62% ± 11% (41–90%)	–	NS
$CD13^{hi}/CD11b^-$ myeloblasts	2% ± 1% (0.2–5.5%)	1% ± 0.5% (0.1–2.5%)	2% ± 1.5% (0.6–8%)	1% ± 0.5% (0.5–2%)	NS
$CD13^{lo/int}/CD11b^-$ promyelocytes	14% ± 6% (3–31%)	7.6% ± 4% (1–19%)	14% ± 6% (6–29%)	8.7% ± 3.5% (4–16%)	NS
$CD13^{lo/int}/CD11b^+$ myelocytes and metamyelocytes	35% ± 10% (12–56%)	19% ± 8% (5–37.5%)	38.5% ± 9% (18–53%)	26% ± 8.5% (11–55%)	<0.03
$CD13^{hi}/CD11b^+$ bands/mature neutrophils	49% ± 13% (27–81%)	33% ± 14% (13–61%)	45% ± 12% (19–65%)	28% ± 10% (12–57%)	NS
Monocytic cells	–	4% ± 2% (0.7–9%)	–	4.5% ± 2% (0–11%)	NS
Nucleated red blood cell precursors	–	15% ± 9% (1–33%)	–	15.5% ± 10% (4–41%)	NS
Eosinophils	–	3.5% ± 4.5% (0.1–20%)	–	2% ± 1% (0.1–5.5%)	NS
Mature lymphocytes	–	13% ± 5.4% (6–27.5%)	–	12% ± 5% (4–23.5%)	NS

Results expressed as mean percentage of cells ± one standard deviation and range between brackets. NS, no statistical significance.

fluorescence in situ hybridization (iFISH) studies were systematically performed on each BM sample, as previously reported, (25) with the following chromosome probes (all purchased from Vysis, Downers Grove, IL) for the detection of the most frequent recurrent abnormalities in patients with MDS: (1) LSI D5S23, D5S71 Spectrum Green (SG)/LSI EGFR Spectrum Orange (SO) probe combination for chromosome 5; (2) LSI D7S486 (7q31) SO/CEP 7 SG probes for chromosome 7; (3) CEP 8 (D8Z2) SO/CEP Y (DYZ1) SG probes for chromosomes 8 and Y, respectively; and (4) LSI D20S108 (20q12) SO probe for chromosome 20. Because the iFISH-searched chromosomal alterations are only present in between 35 and 50% of all MDS cases, investigation of the pattern of inactivation of a chromosome X (HUMARA assay) was analyzed in parallel in FACS-purified BM cell populations (FACSARIA, BDB) (purity $\geq$ 97%) from female individuals, including maturing neutrophils, monocytic cells, NRBC, $CD34^+$ HPC, and mature lymphocytes (identified as described earlier) as described elsewhere (26). Detection of clonality through the HUMARA test is based on the pattern of inactivation of chromosome X in cells from individuals having two X chromosomes.

Statistical Methods

For all variables under study, their mean values and standard deviation, median, and range were calculated using the SPSS software (SPSS 10.0, Chicago, IL). For continuous variables, the Student's *t* test (parametric data) and either the Mann–Whitney *U* or the Kruskal–Wallis tests (nonparametric data) were used in order to investigate the statistical significance of the differences observed among two or more groups, respectively. P values < 0.05 were considered to be associated with statistical significance.

RESULTS

An overall similar distribution of the different compartments of cells investigated was found in normal versus reactive BM samples ($P \geq 0.05$, Table 1). Despite this, detailed analysis of maturing neutrophils showed a significant increase of more immature $CD13^{lo/int}/CD11b^+$ cells (the phenotypic counterpart of myelocytes and metamyelocytes) in reactive versus normal BM ($P = 0.004$) (Table 1). In turn, a similar ($P > 0.05$) overall PI was also found in normal (7% ± 3.6%) versus reactive BM (7.4% ± 3.6%). In addition, no significant differences were found between both groups of BM samples as regards the percentage of $S + G_2 - M$ phase cells among the different BM cell compartments investigated, except for the nonlymphoid $CD34^+$ HPC and monocytic cells, which displayed significantly lower percentage of $S + G_2 - M$ cells in reactive ($P = 0.02$) and normal ($P \leq 0.03$) BM samples, respectively. Noteworthy, NRBC precursors (PI of 28.6% ± 5% vs. 27% ± 6%; $P > 0.05$) followed by the nonlymphoid $CD34^+$ HPC (17% ± 5% vs. 14% ± 5%; $P < 0.03$) displayed significantly higher ($P < 0.001$) percentages of $S + G_2 - M$ cells versus all other BM cell compartments (Table 2) in

Table 2
Proliferation Index (Percentage of S + G_2/M Cells) of Different Cell Compartments in Normal (n = 47) versus Reactive (n = 47) Bone Marrow (BM)

	Proliferation index of BM cell populations (%)				
	Normal BM (*N* = 47)		Reactive BM (*N* = 47)		
	% of specific cell subsets	% of all BM cells	% of specific cell subsets	% of all BM cells	*P*-value
Whole BM	–	7% ± 3.5% (1–17%)	–	7.4% ± 3.5% (2.5–17.5%)	NS
$CD34^+$ HPC					
Nonlymphoid $CD34^+$ precursors	17% ± 4% (9–28%)	0.2% ± 0.3% (0.01–2%)	14% ± 5% (5.6–27%)	0.1% ± 0.06% (0.01–0.3%)	<0.03
Maturing neutrophils	5% ± 2.5% (0.5–11%)	3% ± 1.5% (0.3–7%)	6% ± 3% (2–12)	3.4% ± 1.6% (1.5–9.5)	NS
Monocytic cells	4.6% ± 4.5% (0.1–21%)	0.1% ± 0.1% (0.01–0.6%)	6% ± 3.6% (0.15–15%)	0.3% ± 0.2% (0.02–1%)	0.03
Nucleated red blood cell precursors	28.5% ± 5% (16–37%)	4% ± 2.5% (0.3–9%)	27% ± 6% (11–45%)	4% ± 3% (0.1–14%)	NS
Eosinophils	4% ± 4% (0.0–13%)	0.2% ± 0.5% (0.0–1.8%)	5% ± 3% (0.0–9%)	0.1% ± 0.08% (0.0–0.3%)	NS
Mature lymphocytes	0.0 ± 0.0%	0.0 ± 0.0%	0.0 ± 0.0%	0.0 ± 0.0%	–

Results expressed as mean percentage of S + G_2/M cells ± one standard deviation and range between brackets. NS, no statistical significance.

both normal and reactive BM, respectively. Conversely, intermediate PI was found for the overall population of maturing neutrophils (5% ± 2.5% vs. 6% ± 3%; $P > 0.05$), monocytic cells (4.6% ± 4.5% vs. 6% ± 3%; $P = 0.03$), and eosinophils (4% ± 4% vs. 5% ± 3%; $P > 0.05$) in normal versus reactive BM, respectively; by contrast, mature lymphocytes systematically corresponded to resting cells and showed no $S + G_2 - M$ cells (PI = 0.0% ± 0.0%; $P > 0.05$; Table 2).

Detailed analysis of the different maturation-associated compartments of neutrophil lineage cells, defined by the pattern of expression of CD11b and CD13 (Fig. 2) (27), revealed that $CD13^{hi}/CD11b^-$ (e.g., mainly corresponding to myeloblasts) and $CD13^{lo/int}/CD11b^-$ (e.g., promyelocytes) precursors were those neutrophil-lineage subsets with the highest PI in both normal (22% ± 5% and 21% ± 5%, respectively; $P > 0.05$) and reactive BM (20% ± 6% and 21% ± 5%, respectively; $P > 0.05$) (Table 3). Conversely, the more mature compartments of $CD13^{lo/int}/CD11b^+$ myelocytes and metamyelocytes and $CD13^{hi}/CD11b^+$ bands/mature neutrophils showed significantly lower ($P < 0.001$) percentages of $S + G_2 - M$ cells in both groups of BM samples (Table 3). Interestingly, however, the more mature bands/neutrophils ($CD13^{hi}/CD11b^+$ cells) displayed a greater PI ($P = 0.03$) in reactive (0.9% ± 0.8%) versus normal (0.6% ± 0.5%) BM, in association with increased overall proportion of $CD13^{lo/int}/CD11b^+$ cells (Tables 1 and 3).

DISCUSSION

Assessment of the distribution of hematopoietic cells within a specific cell subset along the G_0/G_1 versus S plus G_2/M cell-cycle phases provides relevant information to monitor growth of normal and leukemic cells (20). Previous studies have shown that the PI of tumor cells is of prognostic value in patients with acute lymphoblastic leukemia, multiple myeloma, B-cell chronic lymphoproliferative disorders, and AML (16,17,28–30). Despite this, a comparison of the PI of distinct BM cell populations in normal versus reactive BM has not been previously reported.

In the present work, we used DRAQ5 to analyze the PI of different BM hematopoietic cell compartments in a relatively large series of normal versus cytopenia-associated reactive BM, as a frame of reference for the definition of altered PI in MDS patients. To discard or include subjects in the reactive BM cohort, we assessed the methylation patterns of the androgen-receptor gene (HUMARA), which has been widely used to determine clonality of cell populations in different types of neoplastic cells (31). Overall, our results confirm and expand on previous observations, (17) which show that among the different compartments of normal BM cells, NRBC, and $CD34^+$ HPC are those cell populations containing the highest number of proliferating cells, while normal mature lymphocytes are typically resting, nondividing cells (17). Interestingly, very similar results were obtained in our and other reports although a slight deviation of PI values in some normal BM cell compartments

Table 3
Mean Proliferation Index (S + G_2/M cells) of Different Maturation-Associated Compartments of Neutrophil Lineage Cells in Normal (n = 47) versus Reactive (n = 47) Bone Marrow (BM)

	Proliferation index of maturation-associated neutrophil cell populations									
	$CD13^{hi}/CD11b^{-}$ cells			$CD13^{lo/int}/CD11b^{-}$ cells			$CD13^{lo/int}/CD11b^{+}$ cells			$CD13^{hi}/CD11b^{+}$ cells		
	% of specific cell subsets	% of all BM cells	% of BM neutrophils	% of specific cell subsets	% of all BM cells	% of BM neutrophils	% of specific cell subsets	% of all BM cells	% of BM neutrophils	% of specific cell subsets	% of all BM cells	% of BM neutrophils
Normal BM	22 ± 5	0.2% ± 0.1%	0.4 ± 0.3	21 ± 5	1.6% ± 1%	3 ± 1.6	4 ± 2	0.8% ± 0.5%	1.4 ± 1	0.6 ± 0.5	0.1% ± 0.1%	0.2 ± 0.2
(*N* = 47)	(13–35)	(0.03–0.6%)	(0.04–1.5)	(12–31)	(0.2–4%)	(0.3–7)	(1–8)	(0.1–2%)	(0.2–4)	(0.1–2)	(0.04–0.5%)	(0.07–0.8)
Reactive BM	20 ± 6	0.2% ± 0.1%	0.4 ± 0.3	21 ± 5	1.9% ± 0.8%	3 ± 1.5	4 ± 2	1% ± 1%	1.6 ± 1	0.9 ± 0.8	0.2% ± 0.1%	0.4 ± 0.2
(*N* = 47)	(4–37)	(0.05–0.6%)	(0.07–1.4)	(12–36)	(0.5–4%)	(0.8–6)	(1.5–12)	(0.3–6%)	(0.3–5)	(0.2–4)	(0.05–0.5%)	(0.1–0.9)
P-value	NS	NS	NS	NS	NS	NS	NS	NS	NS	*0.03*	*0.05*	*0.03*

Results expressed as mean percentage of S + G_2/M-phase cells ± one standard deviation (SD) and range between brackets. NS, no statistical significance.

was observed with respect to those reported by Primo et al. (17), which might derive from the low number of cases analyzed in this latter study. In addition, we extend on previous observations by showing similar PI profiles in normal versus cytopenia-associated reactive BM. Noteworthy, monocytic cells and maturing neutrophils showed intermediate PI in both normal and reactive BM but with higher PI among monocytic cells from reactive versus normal BM. Moreover, despite the overall similar PI of maturing neutrophils in normal versus reactive BM, a more detailed analysis of the different maturation-associated compartments of BM neutrophils also shows a greater PI for the more mature cell compartments. Altogether, our results suggest that a slight shift of proliferation from the early precursors to the more mature granulomonocytic compartment occurs in reactive BM, which could reflect an attempt of the hematopoietic system to rapidly produce functional neutrophils and monocytes, at the expense of a lower expansion of the minor compartments of $CD34^{+}$ HPC. Similarly, such attempt for myeloid expansion has been recently reported by our group to be frequently observed in low-risk MDS in which production of neutrophil lineage cells is essential to counteract the peripheral cytopenias (18,19).

In summary, these results show that in reactive BM, the proliferative index of most BM cell lineages remains unaltered when compared with normal BM, except for a slightly increased PI among monocytic cells and the more mature neutrophil precursors, at the expense of a lower proliferative activity of $CD34^{+}$ HPC. Therefore, an altered PI of specific BM cell populations with respect to those values here reported might contribute (in combination with other phenotypic alterations) to a better diagnostic characterization and classification of clonal myeloid disorders (e.g., MDS) versus reactive BM. Further studies focused on the PI of BM cells from subjects with clonal hematological malignancies at different stages of the disease are necessary to determine the potential utility of cell-cycle studies in the diagnostic and prognostic evaluation of these diseases.

LITERATURE CITED

1. Nurse P. A long twentieth century of the cell cycle and beyond. Cell 2000;100:71–78.
2. Doree M, Hunt T. From Cdc2 to Cdk1: When did the cell cycle kinase join its cyclin partner? J Cell Sci 2002;115:2461–2464.
3. Murray AW. Recycling the cell cycle: Cyclins revisited. Cell 2004; 116:221–234.
4. Bloom J, Cross FR. Multiple levels of cyclin specificity in cell-cycle control. Nat Rev Mol Cell Biol 2007;8:149–160.
5. Budirahardja Y, Gonczy P. Coupling the cell cycle to development. Development 2009;136:2861–2872.
6. Stanger BZ. Organ size determination and the limits of regulation. Cell Cycle 2008;7:318–324.
7. von Wangenheim KH, Peterson HP. The role of cell differentiation in controlling cell multiplication and cancer. J Cancer Res Clin Oncol 2008;134:725–741.
8. Maddika S, Ande SR, Panigrahi S, Paranjothy T, Weglarczyk K, Zuse A, Eshraghi M, Manda KD, Wiechec E, Los M. Cell survival, cell death and cell cycle pathways are interconnected: Implications for cancer therapy. Drug Resist Updat 2007;10:13–29.
9. Dor Y, Melton DA. How important are adult stem cells for tissue maintenance? Cell Cycle 2004;3:1104–1106.

10. van Heemst D, den Reijer PM, Westendorp RG. Ageing or cancer: A review on the role of caretakers and gatekeepers. Eur J Cancer 2007;43:2144–2152.
11. Wells AD. Cyclin-dependent kinases: Molecular switches controlling anergy and potential therapeutic targets for tolerance. Semin Immunol 2007;19:173–179.
12. Montecucco C, Riccardi A, Traversi E, Giordano P, Mazzini G, Ascari E. Proliferative activity of bone marrow cells in primary dysmyelopoietic (preleukemic) syndromes. Cancer 1983;52:1190–1195.
13. Montecucco C, Riccardi A, Traversi E, Danova M, Ucci G, Mazzini G, Giordano P. Flow cytometric DNA content in myelodysplastic syndromes. Cytometry 1983;4:238–243.
14. Peters SW, Clark RE, Hoy TG, Jacobs A. DNA content and cell cycle analysis of bone marrow cells in myelodysplastic syndromes (MDS). Br J Haematol 1986;62:239–245.
15. Vidriales MB, Orfao A, Lopez-Berges MC, Gonzalez M, Lopez-Macedo A, Ciudad J, Lopez A, Garcia MA, Hernandez J, Borrego D, et al. Prognostic value of S-phase cells in AML patients. Br J Haematol 1995;89:342–348.
16. San Miguel JF, Garcia-Sanz R, Gonzalez M, Moro MJ, Hernandez JM, Ortega F, Borrego D, Carnero M, Casanova F, Jimenez R, et al. A new staging system for multiple myeloma based on the number of S-phase plasma cells. Blood 1995;85:448–455.
17. Primo D, Flores J, Quijano S, Sanchez ML, Sarasquete ME, del Pino-Montes J, Gaarder PI, Gonzalez M, Orfao A. Impact of BCR/ABL gene expression on the proliferative rate of different subpopulations of haematopoietic cells in chronic myeloid leukaemia. Br J Haematol 2006;135:43–51.
18. Matarraz S, Lopez A, Barrena S, Fernandez C, Jensen E, Flores J, Barcena P, Rasillo A, Sayagues JM, Sanchez ML, et al. The immunophenotype of different immature, myeloid and B-cell lineage-committed CD34+ hematopoietic cells allows discrimination between normal/reactive and myelodysplastic syndrome precursors. Leukemia 2008; 22:1175–1183.
19. Matarraz S, Lopez A, Barrena S, Fernandez C, Jensen E, Flores-Montero J, Rasillo A, Sayagues JM, Sanchez ML, Barcena P, et al. Bone marrow cells from myelodysplastic syndromes show altered immunophenotypic profiles that may contribute to the diagnosis and prognostic stratification of the disease: A pilot study on A series of 56 patients. Cytometry Part B: Clin Cytometry 2010;78B:154–168.
20. Swerts K, Van Roy N, Benoit Y, Laureys G, Philippe J. DRAQ5: Improved flow cytometric DNA content analysis and minimal residual disease detection in childhood malignancies. Clin Chim Acta 2007;379:154–157.
21. Yuan CM, Douglas-Nikitin VK, Ahrens KP, Luchetta GR, Braylan RC, Yang L DRAQ5-based DNA content analysis of hematolymphoid cell subpopulations discriminated by surface antigens and light scatter properties. Cytometry Part B: Clin Cytometry 2004; 58B:47–52.
22. Sutherland DR, Anderson L, Keeney M, Nayar R, Chin-Yee I. The ISHAGE guidelines for CD34+ cell determination by flow cytometry. International Society of Hematotherapy and Graft Engineering. J Hematother 1996;5:213–226.
23. Alimena G, Billstrom R, Casalone R, Gallo E, Mitelman F, Pasquali F. Cytogenetic pattern in leukemic cells of patients with constitutional chromosome anomalies. Cancer Genet Cytogenet 1985;16:207–218.
24. Mitelman F, editor. An International System for Human Cytogenetic Nomenclature. Basel, Switzerland: Karger; 1995.
25. Ketterling RP, Wyatt WA, VanWier SA, Law M, Hodnefield JM, Hanson CA, Dewald GW. Primary myelodysplastic syndrome with normal cytogenetics: Utility of 'FISH panel testing' and M-FISH. Leuk Res 2002;26:235–240.
26. Teodosio CG-MA, Acevedo MJ, Muñoz LS, Ivan A-T, Nuñez R, Schwarrtz LB, Walls AF, Escribano L, Orfao A. Mast cells from different molecular and prognostic subtypes of systemic mastocytosis display distinct immunophenotypes. J Allergy Clin Immunol 2010;125: 719–726, 726.e1–726.e4.
27. van Lochem EG, van der Velden VH, Wind HK, te Marvelde JG, Westerdaal NA, van Dongen JJ. Immunophenotypic differentiation patterns of normal hematopoiesis in human bone marrow: Reference patterns for age-related changes and disease-induced shifts. Cytometry Part B: Clin Cytometry 2004;60B:1–13.
28. Pui CH, Crist WM, Look AT. Biology and clinical significance of cytogenetic abnormalities in childhood acute lymphoblastic leukemia. Blood 1990;76:1449–1463.
29. Quijano S, Lopez A, Rasillo A, Barrena S, Luz Sanchez M, Flores J, Fernandez C, Sayagues JM, Osuna CS, Fernandez N, et al. Association between the proliferative rate of neoplastic B cells, their maturation stage, and underlying cytogenetic abnormalities in B-cell chronic lymphoproliferative disorders: Analysis of a series of 432 patients. Blood 2008;111:5130–5141.
30. Orfao A, Ciudad J, Gonzalez M, San Miguel JF, Garcia AR, Lopez-Berges MC, Ramos F, Del Canizo MC, Rios A, Sanz M, et al. Prognostic value of S-phase white blood cell count in B-cell chronic lymphocytic leukemia. Leukemia 1992;6:47–51.
31. Rabkin CS, Janz S, Lash A, Coleman AE, Musaba E, Liotta L, Biggar RJ, Zhuang Z. Monoclonal origin of multicentric Kaposi's sarcoma lesions. N Engl J Med 1997;336:988–993.