

# Correction of Pseudoreticulocytosis in Leukocytosis Samples Using the Sysmex XE-2100 Analyzer Depends on the Type and Number of White Blood Cells

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**Background:** The reticulocyte count is a good marker of erythropoietic activity of the bone marrow. In the mid-1990s, automated flow cytometric analysis replaced microscopy for the quantification of reticulocytes. Leukocytosis cases with an erroneously high reticulocyte count and a high immature reticulocyte fraction (IRF) have been reported. In this study, we analyzed reticulocyte counts in leukocytosis samples, in an effort to identify a correction method.

**Methods:** The study comprised of 21 samples from 16 leukocytosis patients. Results of reticulocyte analyses obtained using a XE-2100 hematology analyzer (Sysmex, Japan) were compared with those obtained using the supravital staining technique, which is a reference method. If the samples showed erroneously high reticulocyte counts and IRF, they were reanalyzed after serial dilution with isotonic solution.

**Results:** Five samples from 4 patients showed erroneously elevated reticulocyte counts and/or IRF on the XE-2100 analyzer. They displayed abnormal reticulocyte scattergrams, with 4 of 5 cases indicated by a flag. The white blood cell (WBC) fractions overlapped with the reticulocyte regions, especially with the IRF. Diagnoses and blast counts were variable when such errors occurred; WBC counts varied from  $218.19 \times 10^9/L$  to  $725.14 \times 10^9/L$ . The errors were corrected by simple dilution with isotonic solution. However, the corrective WBC counts differed according to individual cases.

**Conclusions:** When leukocytosis samples exhibit an abnormal reticulocyte scattergram with a flag, or an abnormally high IRF, we recommend the dilution of the sample with isotonic solution to a WBC count of about  $100.00 \times 10^9/L$ , followed by reanalysis of the reticulocyte count and reticulocyte scattergram.

**Key Words:** Reticulocytes, Leukocytosis, Reticulocyte count

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## INTRODUCTION

Reticulocytes are RNA-containing cells that are formed between the erythroblast and mature erythrocyte stages of red blood cell (RBC) maturation. The RNA of RBCs is lost upon cell maturation [1]. The reticulocyte count is a good marker of erythropoietic activity of the bone marrow. It is a useful tool to diagnose

anemia or monitor bone marrow response to therapy [2, 3].

The reference method for reticulocyte enumeration is manual counting by microscopic examination of supravital stained blood smears [4]. In the mid-1990s, automated flow-cytometric analysis replaced microscopic quantitation [5]. Each automatic hematological analyzer adopts different principles for reticulocyte counting [6]. Besides the reticulocyte count, the immature

reticulocyte fraction (IRF) is one of the reticulocyte indices generated by automated analyzers [7]. The IRF is an early predictor of hematological recovery following iron therapy, chemotherapy, or bone marrow transplantation [8-11].

The XE-2100 analyzer (Sysmex, Kobe, Japan) is one of the commonly used instruments for hematological analysis. For reticulocyte enumeration, the XE-2100 analyzer uses fluorescence and light scattering properties. The fluorescent dye polymethine labels the RNA of the reticulocytes, platelets, and WBCs, and the DNA of other nucleated cells [12]. Reticulocytes are distinguished from RBCs on the basis of the fluorescence intensity. The fluorescence intensity is also used to segregate the reticulocyte population into 3 fractions - low, medium, and high fluorescence; the sum of the medium and highly fluorescent reticulocytes represents the IRF [13]. Signals with fluorescence intensity between that of reticulocytes and WBCs represent the upper particle portion (UPP) [14].

Abnormal numbers of WBCs, WBC fragments, nucleated RBCs, giant platelets, and platelet clumps may interfere with automated reticulocyte counting [15]. In our laboratory, for cases where the reticulocyte count is >10% by the automated analyzer and when interfering factors like hyperleukocytosis are present, we verify the count by using the manual reticulocyte counting method. However, the exact WBC count that results in an erroneously high reticulocyte count has not yet been reported. Moreover, a correction method by using the automatic hematology analyzer has not been reported.

Hence, we examined the influence of the WBC count on the reticulocyte count or the IRF by using the XE-2100 hematology analyzer to determine the WBC count for which reticulocytes should be counted manually and the method for correction of the error by using an automated hematology analyzer.

## METHODS

### 1. Reticulocyte counting

Twenty-one blood samples (sample nos. 1-21) were obtained from 16 patients with leukocytosis from November 2011 to February 2012 in Seoul St. Mary's Hospital, Seoul, Korea, and analyzed for complete blood cell (CBC) count (Table 1). Diagnosis was variable and included leukemia and benign non-hematological diseases. WBC counts varied from  $29.64 \times 10^9/L$  to  $725.14 \times 10^9/L$ . The blast counts in the manual WBC differential count ranged from 0 to 99%. The count of nucleated RBC per 100 WBCs ranged from 0 to 1. The reticulocyte count was performed on a XE-2100 hematology analyzer (Sysmex). Reference values

for normal counts in our laboratory were as follows: total reticulocyte count, 0.2-2.0% and IRF, 2.0-15.0%. If the reticulocyte signal overlapped with the WBC signal on the reticulocyte scattergrams, the reticulocyte scattergram was regarded as abnormal; if the reticulocyte signal was well separated from the WBC signal, the scattergram was considered to be normal. An abnormal reticulocyte scattergram prompted the manual enumeration of reticulocytes. For the manual count, an equal volume of peripheral blood anticoagulated with  $K_2EDTA$  was mixed with 1% brilliant cresyl blue solution. Following incubation for several minutes, a thin smear was prepared and each slide was examined using light microscopy. The number of reticulocytes in 1,000 RBCs was determined.

### 2. Correction of error

In case of an abnormal reticulocyte scattergram, the reticulocyte and WBC counts were verified by using the XE-2100 analyzer following serial dilution (1:2-1:10) with an isotonic solution (Sysmex). These reticulocyte counts were compared with the manual counts, and the minimal dilution that resulted in the same reticulocyte count was determined to be the corrective dilution.

## RESULTS

The reticulocyte counts generated by the XE-2100 analyzer in the 21 samples varied from 0.06 to 42.8%. Five samples (nos. 1, 2, 4, 5, and 7) from 4 patients showed erroneously elevated reticulocyte counts on automated counting compared to the manual counts.

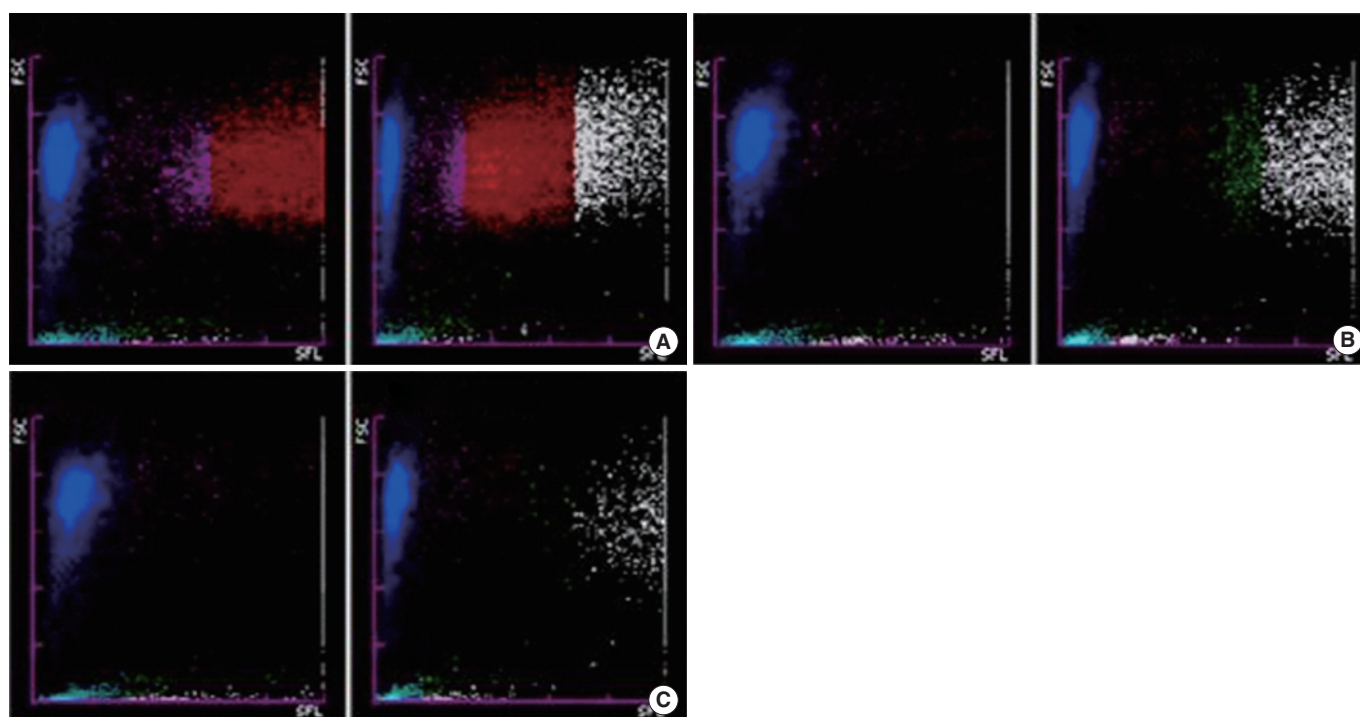
Sample no. 1 was obtained from a patient diagnosed with mixed phenotype acute leukemia (MPAL) with t(v; 11q23); *MLL* rearranged. The WBC count was  $725.14 \times 10^9/L$ . The blast and nucleated RBC counts were 97% and 1 per 100 WBCs, respectively. The automated and manual reticulocyte counts were 42.8% and 1.2%, respectively. The IRF determined using the XE-2100 analyzer was 91.7% (Table 1). The reticulocyte scattergram on the XE-2100 analyzer was abnormal, without the associated "Abnormal reticulocyte scattergrams" flag. The WBC fraction appeared to overlap with the reticulocyte regions, especially with the IRF (Fig. 1). The reticulocyte count, IRF, and the reticulocyte scattergram became normal after a 1:2 dilution, following which, the WBC count decreased to  $380.55 \times 10^9/L$  (Table 1, Fig. 1).

Sample no. 2, which was from the same patient, was obtained 1 day later, following hydration. The WBC count was  $574.55 \times 10^9/L$ . The automated and manual reticulocyte counts were 4.69% and 1.2%, respectively. The IRF was 85.5%, and

**Table 1.** Clinical and laboratory data of reticulocyte analysis in patients with leukocytosis using a XE-2100 analyzer

No. case	No. sample	Sex/Age	Diagnosis	Manual immature WBC count (%)	Dilution factor	WBC (×10 <sup>9</sup> /L)	Reticulocyte analysis using the XE-2100 analyzer				Manual count (%)
							Count (%)	Scattergram	Flag	IRF (%)	
1	1 (day 0)	M/0	MPAL	Blasts 97	ND	725.14	42.8	AbNL	-	91.7	1.2
					1:2	380.55	0.84	NL	-	34.1	1.2
					1:3	273.18	0.86	NL	-	32.8	1.2
	2 (day 1)			Blasts 96	ND	574.55	4.69	AbNL	+	85.5	1.2
					1:3	222.48	0.79	NL	-	24.7	1.2
2	3 (day 0)	F/16	AML	Blasts 99	ND	159.90	0.09	NL	-	11.5	0.1
	4 (day 2)			Blasts 99	ND	264.74	0.63	AbNL	+	81.8	0.1
					1:2	131.72	0.12	NL	-	33.4	0.1
					1:3	90.46	0.14	NL	-	0	0.1
3	5 (day 0)	M/53	CML, CP	Blasts 6	ND	218.19	3.08	AbNL	+	27.7	2.5
				Promyelocytes 5	1:2	118.53	2.99	AbNL	-	42.2	2.5
				Myelocytes 12	1:3	78.56	2.77	AbNL	-	43.4	2.5
					1:10	25.86	2.17	NL	-	23.9	2.5
	6 (day 3)			Promyelocytes 1 Myelocytes 5	ND	108.32	1.22	NL	-	14.6	Not checked
4	7 (day 0)	M/62	CML, BP	Blasts 96	ND	375.72	0.81	AbNL	+	65.8	0.2
					1:2	192.95	0.32	NL	-	25.7	0.2
					1:3	132.84	0.22	NL	-	15.8	0.2
	8 (day 5)			Blasts 98	ND	204.81	0.19	NL	-	19.5	0.1
5	9 (day 0)	M/2	B-ALL	Blasts 98	ND	128.66	0.16	NL	-	7.9	0.1
	10 (day 3)			Blasts 98	ND	299.22	0.16	NL	-	3.8	0.1
6	11	M/65	AML	Blasts 90	ND	117.77	1.29	NL	-	21.2	1.3
7	12	F/44	CML, BP	Blasts 52 Myelocytes 13	ND	113.08	2.26	NL	-	19.7	2.5
8	13	M/48	B-ALL	Blasts 90	ND	101.29	0.69	NL	-	26.9	1.0
9	14	M/62	CMMoL	Blasts 1	ND	104.73	0.44	NL	-	18.2	0.4
10	15	F/16	AML	Blasts 87	ND	81.84	9.23	NL	-	35.6	9
11	16	F/23	AML	Blasts 98	ND	53.57	0.06	NL	-	0	0.1
12	17	M/65	B-ALL	Blasts 81	ND	49.42	0.74	NL	-	21.8	0.8
13	18	F/12	PBSC donor	Myelocytes 2	ND	40.15	1.39	NL	-	20	1.4
14	19	F/61	Autoimmune hepatitis		ND	42.32	2.65	NL	-	15.9	2.8
15	20	M/66	AML*	Blasts 41 Promyelocytes 1 Myelocytes 9	ND	33.90	0.36	NL	-	20.5	0.2
16	21	F/57	SLE		ND	29.64	1.07	NL	-	9.6	0.9

\*AML with t (8; 21)(q22; q22); *RUNX1-RUNX1T1*.Abbreviations: AbNL, abnormal; NL, normal; MPAL, mixed phenotype acute leukemia with t (v; 11q23); *MLL* rearranged; CML, CP, *BCR-ABL1* positive CML, chronic phase; CML, BP, *BCR-ABL1* positive CML, blast phase; B-ALL, B lymphoblastic leukemia with t (9; 22)(q34; q11.2); *BCR-ABL1*; CMMoL, chronic myelomonocytic leukemia; PBSC, peripheral blood stem cell; SLE, systemic lupus erythematosus; WBC, white blood cell; IRF, immature reticulocyte fraction; ND, no dilution.



**Fig. 1.** Reticulocyte scattergrams (left side of each panel) and reticulocyte extended scattergrams (right side of each panel) of sample no. 1 generated by the Sysmex XE-2100 hematology analyzer. (A) Sample no. 1 with no dilution (WBC count,  $725.14 \times 10^9/L$ ) showing abnormal scattergrams. The WBC fractions seem to overlap with reticulocyte regions, especially with the IRF. (B) After 1:2 dilution (WBC count,  $380.55 \times 10^9/L$ ), the reticulocyte scattergram normalized and the overlap between the reticulocyte and WBC regions disappeared. In the reticulocyte extension scattergram, the WBC region shifted to the right, because the fluorescence of WBCs increased. (C) After 1:3 dilution (WBC count,  $273.18 \times 10^9/L$ ), the WBC region shifted further to the right. Regions: blue, mature red blood cells; pink, low fluorescent reticulocytes; red, immature reticulocytes; white, WBCs; green, UPP, upper particle portion. Abbreviations: RET, reticulocyte; RET-EXT, reticulocyte extended; WBC, white blood cell; IRF, immature reticulocyte fraction.

the reticulocyte scattergram was abnormal, with the flag. The erroneously elevated reticulocyte count, IRF, and the reticulocyte scattergram were normalized following a 1:3 dilution, and the WBC count decreased to  $222.48 \times 10^9/L$  (Table 1). The 1:2 dilution was not performed since the sample was insufficient.

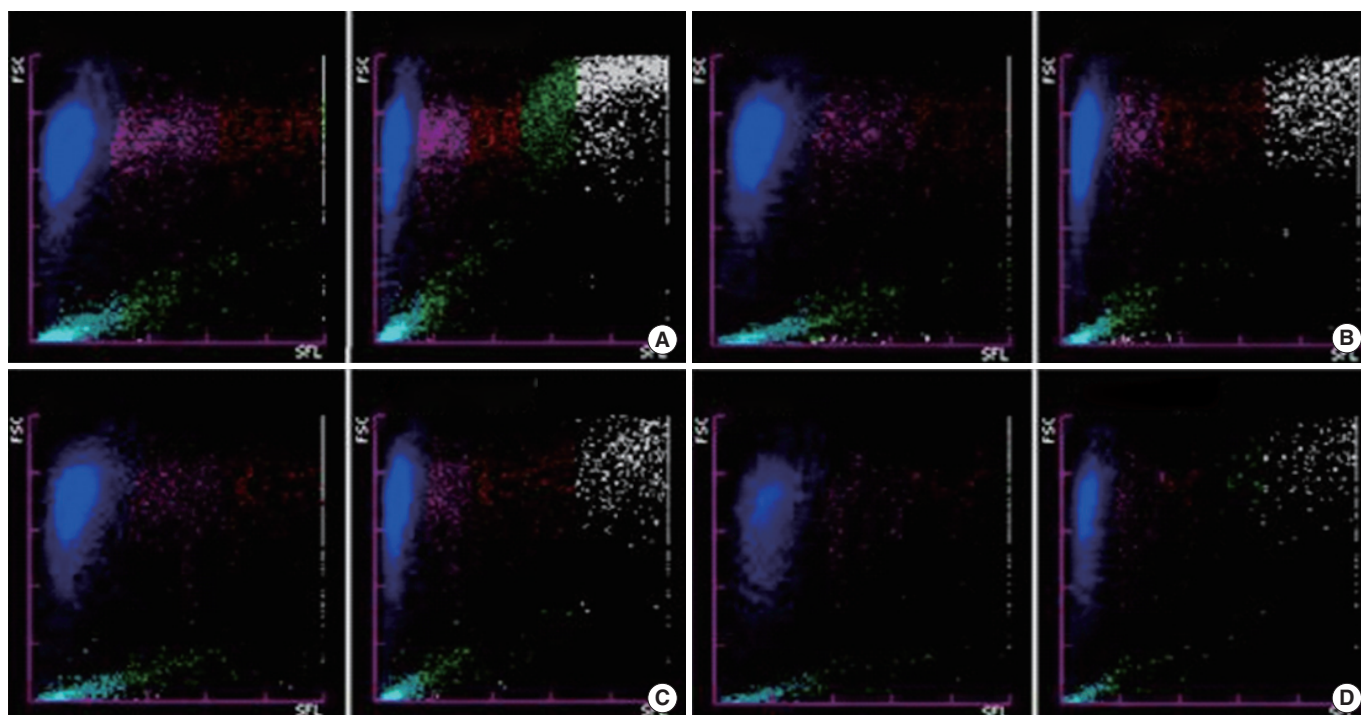
Sample no. 3 was from a patient diagnosed with AML with 99% blasts. The WBC count was  $159.90 \times 10^9/L$ , and the reticulocyte scattergram was normal. The automated and manual reticulocyte counts were 0.09% and 0.1%, respectively, and the IRF was 11.5%.

Sample no. 4 was obtained from the same patient 2 days later following conservative care. The WBC count was  $264.74 \times 10^9/L$ . An erroneously elevated reticulocyte count and IRF were evident, with an abnormal reticulocyte scattergram signaled by the flag. The automated and manual reticulocyte counts were 0.63% and 0.1%, respectively; the IRF was 81.8%. The erroneously elevated reticulocyte count, IRF, and the reticulocyte scattergram were normalized following a 1:2 dilution, which reduced the WBC count to  $131.72 \times 10^9/L$  (Table 1).

Sample no. 5 was obtained from a patient diagnosed with *BCR-ABL1*-positive chronic phase (CP) CML. The WBC count was  $218.19 \times 10^9/L$ . The manual WBC differential count indicated the following: blasts, 6%; promyelocytes, 5%; and myelocytes, 12%. The automated and manual reticulocyte counts were 3.08% and 2.5%, respectively, and the IRF was 27.7%. The flag indicated an abnormal reticulocyte scattergram. The WBC fraction seemed to overlap with the reticulocyte regions, especially with the IRF (Fig. 2A). Even after dilutions of 1:2 (WBC count,  $118.53 \times 10^9/L$ ), and 1:3 (WBC count,  $78.56 \times 10^9/L$ ), the reticulocyte scattergrams remained abnormal, although the WBC region shifted to the right (Fig. 2B, C). After a 1:10 dilution (WBC count,  $25.86 \times 10^9/L$ ), the reticulocyte count and IRF were reduced to 2.17% and 23.9%, respectively. The reticulocyte scattergram was normalized without the flag (Fig. 2D).

Sample no. 6 was from the same patient as sample no. 5, but was obtained following leukapheresis and hydroxyurea treatment. The WBC count decreased to  $108.32 \times 10^9/L$ , and the reticulocyte scattergram was normal (Fig. 3B). The reticulocyte





**Fig. 2.** Reticulocyte scattergrams (left side of each panel) and reticulocyte extended scattergrams (right side of each panel) of sample no. 5 generated by the Sysmex XE-2100 hematology analyzer. (A) Sample no. 5 with no dilution (WBC count,  $218.19 \times 10^9/L$ ) showing abnormal scattergrams. The WBC fractions seem to overlap with reticulocyte regions, especially with the IRF. (B) After 1:2 dilution (WBC count,  $118.53 \times 10^9/L$ ), the scattergrams were still abnormal. However, in the reticulocyte extended scattergram, the WBC region shifted to the right, because the fluorescence of WBCs increased. (C) After 1:3 dilution (WBC count,  $78.56 \times 10^9/L$ ), the scattergrams were still abnormal. (D) After 1:10 dilution (WBC count,  $25.86 \times 10^9/L$ ), the scattergrams were normalized. Regions are the same as described in Fig. 1. Abbreviations: RET, reticulocyte; RET-EXT, reticulocyte extended; WBC, white blood cell; IRF, immature reticulocyte fraction.

count and IRF were 1.22% and 14.6%, respectively, on the XE-2100 analyzer.

Sample no. 7 was obtained from a patient diagnosed with blast phase CML. The WBC count was  $375.72 \times 10^9/L$ . Manual WBC differential count revealed 96% blasts. The automated and manual reticulocyte counts were 0.81% and 0.2%, respectively, whereas the IRF was 65.8%. The reticulocyte scattergram was abnormal as indicated by the flag. Following a 1:2 dilution (WBC count,  $192.95 \times 10^9/L$ ), the reticulocyte count and IRF decreased to 0.32% and 25.7%, respectively. The reticulocyte scattergram was normalized without the flag.

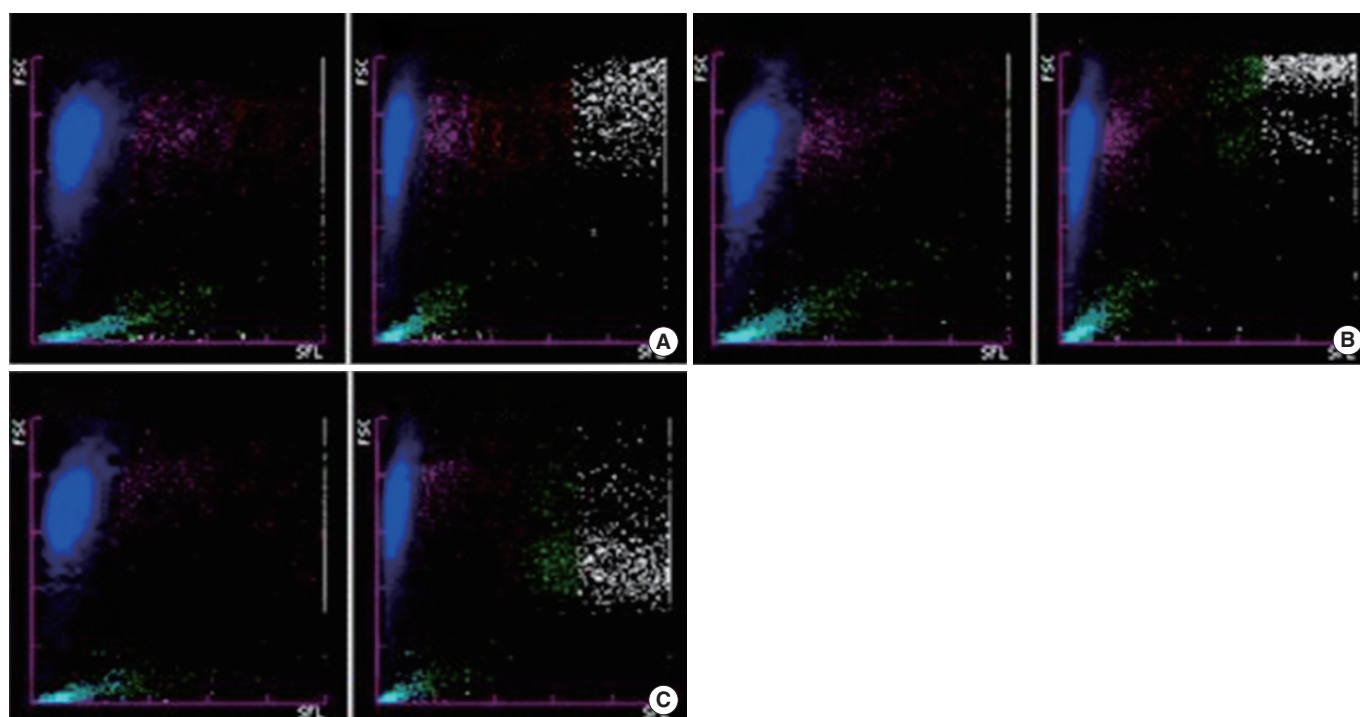
Sample no. 8 was obtained from the same patient as sample no. 7 following hydroxyurea treatment. The WBC count was reduced to  $204.81 \times 10^9/L$ , and the reticulocyte scattergram was normal. The automated and manual reticulocyte counts were 0.19% and 0.1%, respectively. The IRF was 19.5%.

Comparison of the results obtained from the XE-2100 analyzer and from the reference method revealed that 13 samples (nos. 9-21) with a WBC count ranging from  $29.64 \times 10^9/L$  to  $299.22 \times 10^9/L$  displayed the correct reticulocyte count (Table 1). Of

these, 6 displayed WBC counts of  $>100.00 \times 10^9/L$ ; the corresponding diagnoses varied from hematological malignancy to benign non-hematological disease, and the blast counts also showed variation. The 13 samples displayed normal reticulocyte scattergrams. Automated counting resulted in a reticulocyte count varying from 0.06 to 9.23%. Since the difference between manual and automated reticulocyte counts fell mostly within acceptable limits ( $<10\%$  of mean reticulocyte count), we did not dilute the samples.

## DISCUSSION

The present study describes 5 leukocytosis cases associated with erroneously elevated reticulocyte counts, IRF values, and abnormal reticulocyte scattergrams obtained by using the Sysmex XE-2100 hematology analyzer. The samples differed with regard to patient diagnoses, WBC counts, and blast counts. However, all of them displayed an abnormal reticulocyte scattergram, indicated by flag messages in certain cases. The flag messages seemed to be less sensitive than abnormal reticulo-



**Fig. 3.** Reticulocyte scattergrams (left side of each panel) and reticulocyte extended scattergrams (right side of each panel) of samples nos. 5, 6, and 13 generated by the Sysmex XE-2100 hematology analyzer. Sample nos. 5, 6, and 13 had similar WBC counts ( $\sim 100.00 \times 10^9/L$ ), but showed normal or abnormal scattergrams. (A) After 1:2 dilution of sample no. 5 (WBC count,  $118.53 \times 10^9/L$ ), scattergrams were abnormal. (B) Normal scattergrams of sample no. 6 (WBC count,  $108.32 \times 10^9/L$ ). (C) Normal scattergrams of sample no. 13 (WBC count,  $101.29 \times 10^9/L$ ). Regions are the same as described in Fig. 1.

Abbreviations: RET, reticulocyte; RET-EXT, reticulocyte extended; WBC, white blood cell.

cyte scattergrams. In the reticulocyte and extended reticulocyte scattergrams, the WBC fractions appeared to overlap with the reticulocyte regions, especially with the IRF. This could possibly reflect the insufficiency of the fluorescent dye in samples with numerous nucleated cells. Abnormal numbers of WBCs, WBC fragments, nucleated RBCs, giant platelets, and several other factors have been reported to lead to inaccurate gating of RBCs and are hence regarded as possible factors interfering with automated methods for reticulocyte analysis [16-19]. In leukemia patients, erroneously elevated immature reticulocyte counts obtained by using the XE-2100 analyzer have also been reported [14]. In this study, reduction of the WBC counts by simple dilution with an isotonic solution was able to address the abnormal scattergrams. The WBC region shifted to the right, as the fluorescence intensity increased. However, the highest WBC count with an accurate reticulocyte count and normal scattergram differed between cases. A small number of cases showed erroneously elevated reticulocyte counts with a WBC count of  $< 100.00 \times 10^9/L$ , whereas certain others showed normal reticulocyte counts and scattergrams despite a WBC count of  $380.00 \times 10^9/L$ . Therefore, the degree of WBC labeling with the fluorescent dye

polymethine differed among the WBCs, especially in samples from leukemia patients. It should be noted that the WBC count and the type of WBC affected reticulocyte enumeration by the automatic analyzer. Although the CML sample could not be corrected up to a WBC count of  $70.00 \times 10^9/L$ , most cases with an erroneously elevated reticulocyte count determined using the Sysmex XE-2100 system were corrected when samples were diluted to a WBC count of about  $100.00 \times 10^9/L$ . This difference may be associated with the differential labeling of blasts and CML cells by polymethine.

In conclusion, when leukocytosis samples display an abnormal reticulocyte scattergram flag or an abnormally high IRF, we recommend dilution of the samples with isotonic solution to a WBC count of about  $100.00 \times 10^9/L$ , followed by reanalysis of the reticulocyte count as well as the scattergram.

### Authors' Disclosures of Potential Conflicts of Interest

No potential conflicts of interest relevant to this article were reported.

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