original paper
Haematologica 1994; 79:420-428

DIAGNOSIS OF ACUTE MYELOID LEUKEMIA AND SYSTEM COULTER VCS

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ABSTRACT

Background. **The aim of this study was to evaluate the possible contribution VCS could make in a hematological laboratory for the diagnosis of acute myeloid leukemia (AML).**

Materials and Methods. **Peripheral blood samples from 42 AML patients and 58 normal donors were analyzed by flow cytometry with the VCS. Normal and leukemic peripheral blood samples were tested to establish a correlation between VCS data and the reference manual method. We evaluated the sensitivity threshold of the VCS for blast cell detection in progressively diluted samples. We looked at a correlation between different scatterplots and flags and the FAB classification of acute myeloid leukemia in order to identify a characteristic VCS image for each subtype. Thirty-four bone marrow samples (18 normal donors and 16 leukemic patients) were analyzed by the VCS system to demonstrate a characteristic scattergram distribution. Further, we tried to compare scatterplots to the flags of leukemic bone marrow samples and, finally, we compared VCS scatterplots with aberrant antigen expression in AML cases.**

Results and Conclusions. **Overall VCS specificity was 93.1% (54/58) in peripheral blood samples; sensitivity was 100% (42/42) and VCS efficiency was 96%. In AML the characteristic X6 flag was observed in 95.23% of the cases (40/42). In peripheral samples discrimination was made between AML M1 with agranular blasts >50% of the non erythroid cells (NEC), M4, M5 on the one hand, and AML M1 with granular blasts >50% of NEC, M2, M3 on the other: the X5 flag was often present in the second group because of the different localization of the cells (p = 0.001). In all normal bone marrow samples we observed granuloblasts in different maturation stages in the neutrophil region of the DF1 VCS scatterplot corresponding to the X5X6 flags or, rarely, to the X5X6X1, because of the presence of immature erythroid cells. This association X5X6 was never observed alone in patients affected by AML. In our study, it was difficult to identify peculiar scatterplots and alarms for each FAB class of AML. Nevertheless, we observed that in all M4 and M5 FAB cases the blastic cells both in the peripheral blood and in the bone marrow samples were located in the monocyte region, with the frequent presence of the X3 flag often associated with the X6 flag. Eight out of the 16 AML bone marrow samples (1 FAB M0, 1 M2, 1 M3, 2 M4, 3 M5) showed the X2 flag and partial localization of blasts in the lymphoid region. In all these cases the presence of some small blastic cells with agranular cytoplasm was confirmed by morphological observation and cytochemical stainings.**

Key words: VCS system, acute myeloid leukemia, flow cytometry

The Coulter VCS is a flow cytometer that can be used to obtain differential multidimensional leukocyte screening. By employing a three-system combination such as volume, conductivity and laser light scatter, a true representation of different cell population distributions can be shown without any evidence of cellular trauma or reagent collapsing. Moreover, Coulter VCS has an alarm system marked from X1 to X7 to alert the operator when morphological differences arise.[1-4] The Coulter VCS has been used for correlations

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Acknowledgments: the authors wish to thank Dr. R. Caporale, Dr. S. Ciolli and Prof. P.L. Rossi Ferrini for their critical revision of the manuscript.
Received April 11, 1994; accepted July 18, 1994.

with manual cell differential counts,[5,6] and a comparison with other automated counters was obtained and evaluated.[2,4] The object of this study was to evaluate the VCS flow cytometer contribution in the diagnosis of acute leukemia. This preliminary investigation focused on five points: 1) evaluation of VCS efficiency in discriminating a normal peripheral blood sample from a leukemic one; 2) evaluation of VCS sensitivity in giving evidence of blast cells in diluted blood samples; 3) evaluation and interpretation of alarm signals, i.e. correlation between VCS data and morphological aspects in leukemic peripheral blood samples; 4) characterization of normal bone marrow samples and comparison between normal and leukemic marrows; 5) correlation between alarms and immunophenotype.

Materials and Methods

Coulter VCS

The Coulter VCS flow cytometer gives a leukocyte differential count using 100 μL of blood. This instrument is able to count a large number of cells (8192) in a short time (from 1 to 89 seconds). The fundamental principles of the VCS are as follows:

– a volumetric analysis (Coulter principle) deriving from the impedance of a low-frequency current (V = volume).
– a structural analysis related to cellular conductivity for high frequency current (22 kHz) application. Information is also provided regarding internal cellular content (C = conductivity).
– a light dispersion analysis with a red neon-helium laser ray (633 nm emission wave length) evaluates the surface and inner structure of cells and provides supplementary details about size, shape and reflectivity[3,7,8] (S=scatter light).

Different scatterplots are available for the operator. The DF1 (*discriminant function 1*) scatterplot derives from the light scatter where volume is represented on the Y-axis and scatter on the X-axis. Lymphocytes, monocytes, neutrophils and eosinophils are the most prominent populations. A color code shows the population density in decreasing order: yellow indicates the largest concentration of particles (>11 cells) with red (5-10 cells), green (2-4 cells) and blue (1 cell). VCS is equipped with an alarm signal system to alert the operator when morphologic abnormalities exist. The message NEGATIVE is shown when a sample exhibits normal distribution. The message POSITIVE is shown when an irregular pattern of cell distribution is identified. One or more of the seven "X" flags labeled X1 through X7 will accompany a report with a POSITIVE message. The flags are not triggered by quantitative irregularities in cell populations but by qualitative ones.[3,8]

From November 1992 to February 1994, 58 peripheral blood samples from normal donors and 42 (21 females and 21 males, median age: 48 yrs) from patients affected by de novo acute myeloid leukemia were evaluated. Twenty patients with acute lymphoid leukemia (ALL) were also analyzed. These peripheral blood samples were examined either by the reference manual method or by the Coulter VCS. VCS specificity, sensitivity, predictive values and efficiency

Table 1. Equations calculated for predictive value and efficiency of the Coulter VCS.

Specificity	⇒	VCS capacity to correctly settle a normal sample (TN/TN+FP)×100
Sensitivity	⇒	VCS capacity to correctly settle a pathological sample (TP/TP+FN)×100
Predictive value of a positive test	⇒	VCS capacity to discriminate between a pathological and a normal sample (TP/TP+FP) ×100
Predictive value of a negative test	⇒	VCS capacity to discriminate between a normal and a pathological sample (TN/TN+FN) ×100
Efficiency of VCS	⇒	(TP+TN/TP+FP+FN+TN) ×100

TN: true negative. Absence of flag and abnormal cells. FN: false negative. Absence of flag and presence of abnormal cells.
TP: true positive. Presence of flags and abnormal cell. FP: false positive. Presence of flags and absence of abnormal cells

in flagging for the presence of blast cells was determined[9] (Table 1).

VCS system sensitivity in revealing blast cells was evaluated by diluting peripheral blood samples. To accomplish this we used a leukemic peripheral blood sample with 100% of blast cells and added autologous plasma at scalar dilutions (1:2; 1:4; 1:8; 1:16; 1:32; 1:64 etc). Starting with a POSITIVE message and flags X2X3X6 on the VCS, we observed the results with each dilution until a NEGATIVE message appeared. Bone marrow blood was obtained by aspiration and anticoagulated with heparin. The samples (18 normal and 16 from AML patients), which included bone marrow particles, were not diluted with isotonic solution or manipulated mechanically, they were processed directly through the VCS. Cytochemical staining of bone marrow and blood smears was performed by standard methods that included PAS, peroxidase, Sudan black B, naphthol ASD chloroacetate esterase, α-naphthyl acetate and α-naphthyl butyrate esterases, acid phosphatase.[10]

In order to determine the immunophenotype, mononuclear cells were separated using a density gradient (1077 g/mL) Ficoll-Hypaque (Nycomed as., Oslo, Norway), washed three times in phosphatase buffered saline with 0.2% bovin serum albumin and 0.2% sodium azide (PBSA), and then resuspended at a final concentration of 10^6/mL. Afterwards, the cells were incubated at 4°C with several monoclonal antibodies (MoAbs) conjugated with fluorescein isothyocyanate (FITC) or phycoerythrin (PE) for 20-30'. After two washings with PBSA, the cells were run through the flow cytometer (Epics Profile Coulter, Hialeah, FL, USA). Surface antigens were analyzed with the following MoAbs: anti-Calla FITC (CD10), Leu12 FITC (CD19), Leu16 FITC (CD20), Leu5b FITC (CD2), Leu4 FITC (CD3), Leu1 FITC (CD5), Leu9 FITC (CD7), anti-HLA-DR PE, HPCA-2 FITC (CD34), LeuM1 FITC (CD15) purchased from Becton-Dickinson (Mountain View, CA,USA); My7 PE (CD13), My9 PE (CD33), My4 FITC (CD14), NKH-1 PE (CD56) obtained from Coulter (Hialeah, FL, USA).

An indirect immunofluorescence assay using a polyclonal rabbit serum (Supertechs, Bethesda, MD, USA) was performed on cytospin smears for TdT detection, as previously described.[11] Statistical analysis was carried out using the chi-square test.

Results

Evaluation of VCS efficiency in discriminating a normal peripheral blood sample from a leukemic one

When we analyzed peripheral blood samples we observed that the instrument distinguished normal samples with a specificity equal to 93.1%. Four cases which were normal for the reference method but which triggered a POSITIVE message with the VCS were observed. In these cases the X5 flag was isolated and found to correspond to moderate neutrophilosis with the presence of band cells.[6] The sensitivity of the VCS was 100%. In fact, in the 42 leukemic cases the VCS was able, using the proper flags, to give evidence of morphologically abnormal cells in all peripheral blood samples. No NEGATIVE message was observed. The predictive value of a positive test and of a negative test was equal to 91.3% and 100%, respectively. The efficiency of the VCS was 96% (Table 2).

Dilution tests

The sensitivity of the VCS in quantifying and selecting blasts from among other morphologically different cells was evaluated by adding autologous plasma to scalar dilutions of peripheral blood from a patient affected by acute leukemia with 100% of blasts. Blasts were detected at the lowest concentration of 200/μL.

Table 2. Comparison between Coulter VCS and reference method analysis of normal and pathological blood samples.

		Reference method manual		
		Normal	*Pathological*	*Total*
VCS	NEGATIVE	TN=54	FN=0	54
	POSITIVE	FP=4	TP=42	46
	TOTAL	58	42	100

Evaluation and frequencies for each alarm signal

When peripheral blood from 42 leukemic patients was evaluated the following flags were observed (Table 3):

– the X1 flag appears when there is a shifting of the leukocyte analysis threshold, especially in the presence of unlysed erythrocytes, erythroblasts, giant platelets and debris. The X1 flag linked with other flags was shown in 5/42 AML cases;
– the X2 flag is a floating discriminator between the lymphocyte and monocyte regions. Normally it indicates an atypical population in the upper quadrant of the lymphocyte region. The X2 flag alone was observed in ALL cases presenting large non granular blasts, thus moving the analysis threshold between lymphocytes and monocytes upward. The X2 flag appeared in 1 AML M5 with Sudan Black B and peroxidase < 3%. The X2 flag was also seen together with other flags in 10 AML cases;
– the X3 flag is a floating discriminator between the monocyte and neutrophil regions. The X3 flag alone appeared in 1 AML M1 case because of the presence of large agranular elements. In this case the threshold between monocytes and lymphocytes moved to the right. This flag was linked with others in 71.42 % of the cases (30/42) evaluated;
– the X4 flag is a floating discriminator between the lymphocyte and neutrophil regions. The X4 flag was never observed either alone or linked with others;
– the X5 flag is an alarm corresponding to the upper area in the neutrophil region showing immature elements of the granulocytic line or hypersegmented neutrophils. The X5 flag was observed never alone, but only together with other signals in 42.85% (18/42) of the cases evaluated;
– the X6 flag is a tri-regional alarm which appears in the lymphocyte or in the monocyte or the neutrophil area. It indicates the presence of immature cells. The X6 flag was observed in 95.23% of the subjects (40/42): alone in 5/42 cases and linked with other flags in 35/42;
– the X7 flag indicates a morphological abnormality in the lower area of the lymphocyte region. The X7 flag was observed never alone, but only together with other flags in 2 cases of AML M5.

VCS data and morphological aspects for each AML subtype (Table 4)

ALL (data not shown): in a DF1 scatterplot a large homogeneous population was observed in the lymphocyte area, moving it upward due to its greater volume and the presence of agranular elements. The most represented flags were X2 (16/20) and X6 (16/20) (Figures 2a and 2b).

AML M0: in the DF1 scatterplot we observed a population located in either the neutrophil or the lymphocyte area, because of agranular blasts (Sudan Black B and peroxidase <3%). The flags were X2X3X5X6.

AML M1: (10 cases) scatterplots showed two different kinds of figure. In the first one (M1a), evidence was given of a population with a few granular cells located in the lymphocyte and monocyte regions (X3 and X6 flags); in the second one (M1b), the granulated cells were located in the neutrophil region (X3X5X6 flags) (Figures 3a, 3b, 3c).

AML M2: the most represented flags were the

Table 3. Flag distribution in analysis of peripheral blood from patients affected by AML

		Morphological cytotype FAB criteria						
		M0	M1	M2	M3	M4	M5	Total
	X2	—	—	—	—	—	1	1
	X3	—	1	—	—	—	—	1
	X6	—	1	1	1	1	1	5
	X2X6	—	1	—	—	—	—	1
	X3X6	—	3	—	2	3	2	10
F	X5X6	—	—	—	1	1	—	2
L	X1X3X6	—	—	—	—	—	1	1
A	X1X5X6	—	—	—	—	1	—	1
G	X2X3X6	—	—	—	—	2	1	3
S	X2X5X6	—	—	1	—	—	—	1
	X3X5X6	—	3	1	1	—	1	6
	X3X6X7	—	—	—	—	—	2	2
	X1X3X5X6	—	—	3	—	—	—	3
	X2X3X5X6	1	1	3	—	—	—	5
	Total	1	10	9	5	8	9	42

Table 4. Flag distribution in bone marrow samples.

		morphological cytotype FAB criteria						
		M0	M1	M2	M3	M4	M5	Total
	X6	—	—	1	—	1	—	2
	X2X3	—	—	1	—	—	—	1
F	X2X6	—	—	—	—	1	1	2
L	X3X6	—	—	—	1	2	1	4
A	X2X3X4	1	—	—	—	—	—	1
G	X2X3X6	—	—	—	1	1	2	4
S	X3X6X7	—	1	—	—	—	—	1
	X1X3X5X6	—	—	1	—	—	—	1
	Total	1	1	3	2	5	4	16

X6 (9/9), X5 (8/9) and X3 (7/9). Medium-large blast cells with cytoplasmic azurophilic granules were present in large proportion in the neutrophil region (Figures 4a and 4b).

AML M3: the presence of atypical promyelocytes was observed with light microscopy, and VCS allowed us to show the position of these elements in the DF1 scatterplot. The cells often fell in the neutrophil region. The most signaled alarms were X6 (5/5) and X3 (3/5) (Figures 5a and 5b).

AML M4: with VCS evidence of monocytic blasts such as a homogeneous voluminous population, some with granules, appeared in the monocyte area. X6 and X3 were the most represented flags: 8/8 for the former and 5/8 for the latter (Figures 6a and 6b).

AML M5: the X6 and X3 flags were observed in 8 and 7 cases, respectively. The association X3X6, alone or linked with other alarms, was noted in 7 cases. Peripheral and bone marrow blasts exhibited non-specific esterase activity that was inhibited by sodium fluoride. These cells shifted to the monocyte region and moved the X3 floating discriminator to the right (Figures 7a and 7b).

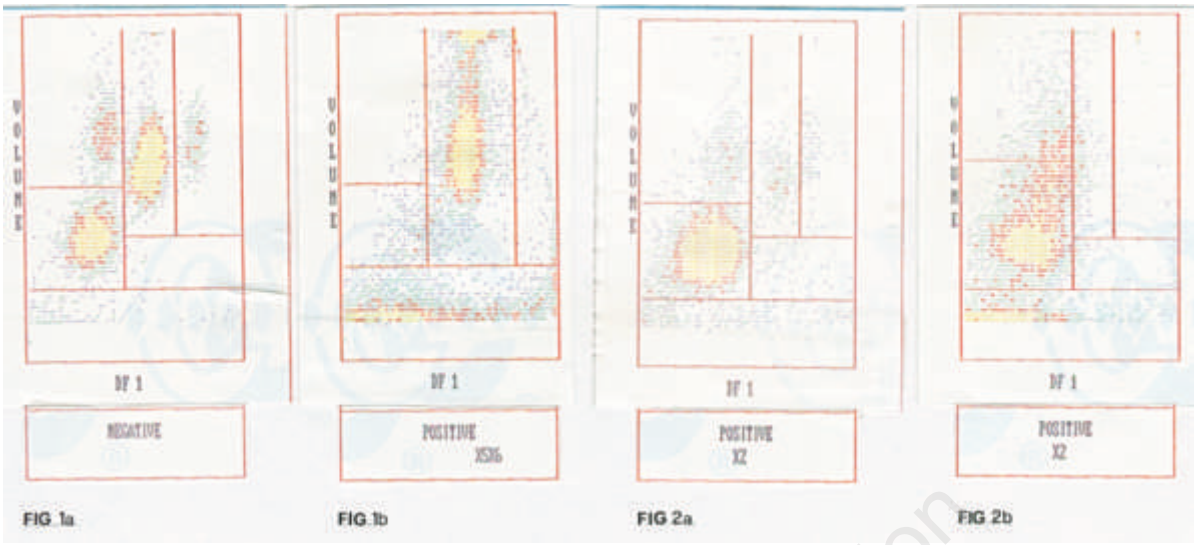
Comparison between normal and pathological bone marrow

The normal bone marrow picture is constantly represented by the X5X6 flags, indicating the presence of granular cells in the upper area of the neutrophils, and by signals of immature cells. Of all the graphic representations available on the VCS, the cellular distribution aspect on scatterplot DF1 was shown to be the most discriminative one. Moreover, in nearly all the cases evaluated, the typical and characteristic image of normal bone marrow was a homogeneous cloud whose base was wider than its top. This particular cell distribution and the X5X6 signal are related to the presence of granuloblasts in different stages of maturation. Sometimes we noted the X1 flag linked with the X5X6 due to the presence of some erythroblasts, which moves the analysis threshold upward. In pathological bone marrow, the arrangement of blasts is very important, and is related to size, cytoplasmic granules and surface characteristics. In 16 bone marrow samples of AML patients, the X5X6 flags were never observed alone. Since the Coulter VCS indicates normal bone marrow samples with a POSITIVE message and the flags X5X6 or X5X6+X1, it was difficult to correlate the different alarms with each subtype of acute leukemia. Nevertheless, when we evaluated the DF1 scatterplot we observed that in AML M4 (5 cases) and M5 (4 cases) the blastic cells were located in the monocyte region (Figure 1b).

Correlation between alarms and immunophenotype

Leukocyte differential analysis performed with the VCS on bone marrow samples from AML patients allowed us to observe different flags, in particular X2, in 8 cases with blasts up to 95%. At first we tried to explain the triggering of this flag, specific for lymphoid or simil-lymphoid cell abnormalities, optically. A high percentage of these elements (50%) was observed in one case: a patient originally affected by B chronic lymphocytic leukemia that subsequently evolved into a minimally differentiated acute myeloid leukemia (AML M0).

In the other AML cases with X2 flag, the lymphocyte percentage was low (around 2%). In such cases we observed the presence of some small blast cells with scant agranular cytoplasm. In 5 out of 8 cases the immunological phenotype showed markers belonging to both the

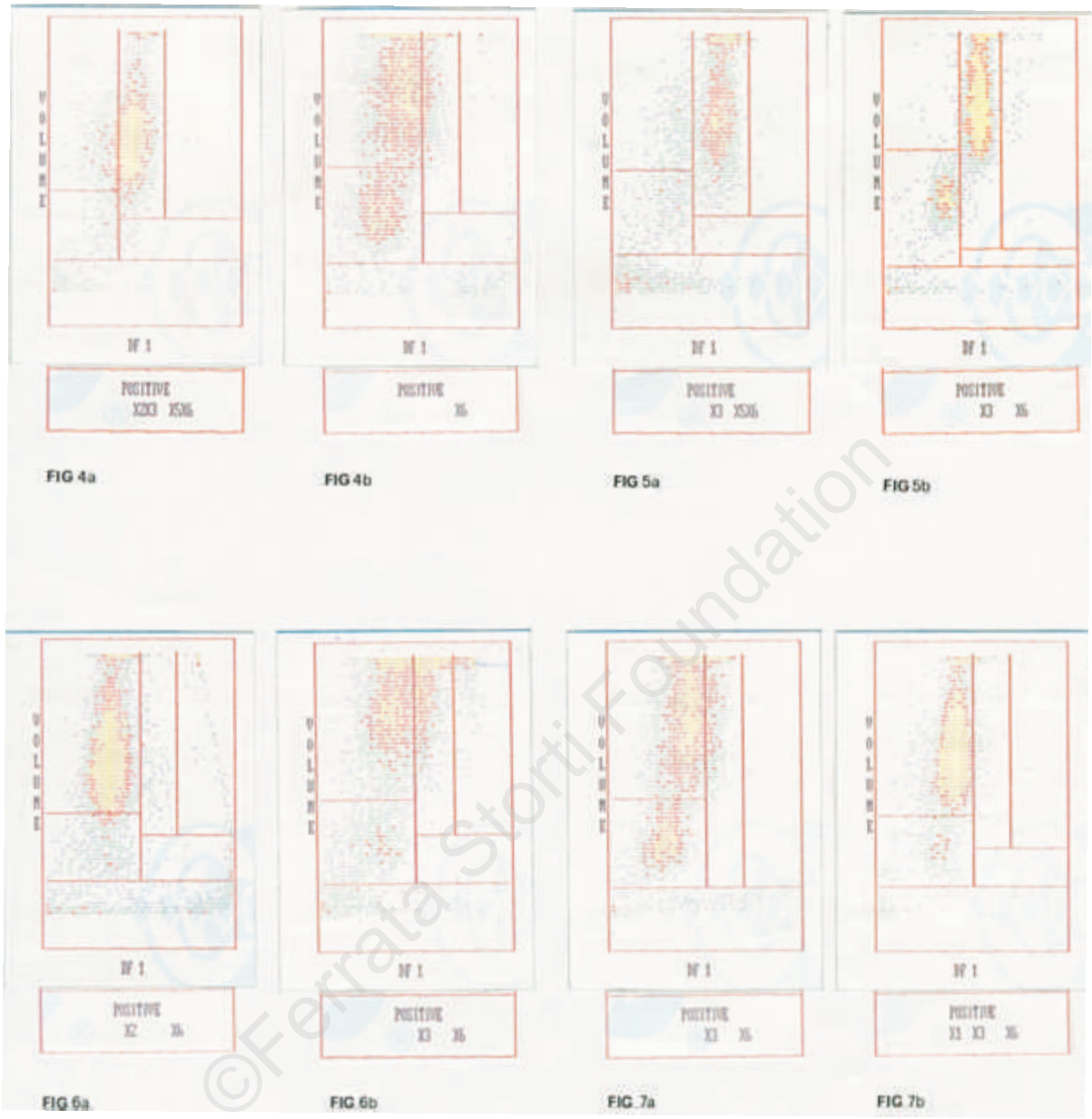


Figures 1-3. 1a: normal peripheral blood sample; 1b: normal bone marrow sample; 2a: ALL peripheral blood sample; 2b: ALL bone marrow sample; 3a: AML M1a peripheral blood sample; 3b: AML M1b peripheral blood sample; 3c: AML M1 bone marrow sample.

myeloid and the B and/or T cell lineages (Tables 5 and 6).

Discussion

Now that flow cytometry has been applied to blood counters for an *automated differential leukocyte count*, the laboratory analyst has access further information that permits more accurate identification of pathological samples and better definition of cellular populations.[12-14] The Coulter VCS has been evaluated previously by



Figures 4-7. 4a: AML M2 peripheral blood sample; 4b: AML M2 bone marrow sample; 5a: AML M3 peripheral blood sample; 5b: AML M3 bone marrow sample; 6a: AML M4 peripheral blood sample; 6b: AML M4 bone marrow sample; 7a: AML M5 peripheral blood sample; 7b: AML M5 bone marrow sample.

several investigators, especially regarding comparisons with the optical method.[2,5,6,7,12,15]

In our study, we observed that the VCS significantly discriminates a normal peripheral blood sample from a leukemic one (sensitivity was 100%). The VCS detected the blast cells in all leukemic cases (42/42) and signalled them not only by a POSITIVE message but also by flags. Thanks to the X6 flag (40/42 cases), the operator can very quickly identify the presence of these pathologic cells. As reported by Picard et al.[6] the combination X3X6 was often found in acute

leukemia. In fact, X3X6, alone or linked to other flags, was observed in 66.66% of the cases evaluated (28/42). In order to determine the sensitivity threshold of VCS, we performed dilution tests and obtained a value of 200 blasts/μL. Accordingly, this instrument can be used in association with an automated blood counter when white blood cell abnormalities are observed in the histogram.[2,6] Regarding the comparison between FAB classification[16] and VCS data, we conclude that:

– lymphoid blasts, irrespective of size, were moved to the lymphocyte region with signals of the X2 or X6 or X2X6 flags;
– the blasts situated in the monocyte and lymphocyte regions are immature myeloblasts (not granulated) or monocytic blasts. The most evidenced alarms were X2,X3,X6, alone or linked together;
– granular blasts and atypical promyelocytes were often shifted to the neutrophil region. X5,X6,X3, alone or linked together, were the most often triggered flags. Accurate observation of the DF1 scatterplot, analysis of the distribution of the cellular population in the different regions and the presence of some specific flags allowed a preliminary discrimination between AML M1a, M4, M5 on the one hand, and AML M1b, M2, M3 on the other.[17] In all AML M1a, M4 and M5 cases, the blast population was positioned in the lymphocyte and monocyte regions. In most cases of AML M1b, M2 and M3, the blasts and the promyelocytes were moved to the neutrophil region. A further distinction between AML M1a, M4, M5 and M1b, M2, M3 was the presence of the X5 flag in 13.04% (3/23) and 77.77% (14/18) of the cases, respectively, with a statistically significant difference between the two groups (p=0.001). Normal bone marrow was identified by the VCS (18 cases were run through the Coulter VCS), and it was necessary to identify the presence of distinctive flags and the scatter of different cellular populations under normal conditions in order to compare it in a second instance with leukemic bone marrow samples.

In our study it was difficult to determine the specificity of each type of alarm in leukemic bone marrow samples. In normal marrow the presence of granuloblasts and immature erythroid cells was signalled by X5X6, and sometimes by X5X6+X1, with a homogeneous cloud whose base was wider than its top.[18] Nevertheless, we observed that:

– in ALL (data not shown) the blasts were located in the lymphocyte region and the X2 and/or X6 flags were seen.
– in the AML cases studied the presence of the X2 and/or the X3 flag often appeared to be linked with the X6 flag. Another difficulty was encountered when leukemic peripheral blood and bone marrow samples of the same patients were analyzed by the VCS. Different distributions were observed on the scatterplots, probably due to physico-chemical dif-

Table 5. Immunologic phenotype in bone marrow samples with the X2 flag.

cytotype flags		*immunologic phenotype*							
		CD10	*CD19*	*CD20*	*CD2*	*CD3*	*CD5*	*CD7*	*Tdt*
AML M0	X2X3X4	2	51	46	5	5	29	6	+
AML M2	X2X3	22	4	2	24	1	26	76	+
AML M3	X2X3X6	2	3	3	10	7	3	20	–
AML M4	X2X6	1	1	2	23	21	6	23	+
AML M4	X2X3X6	15	1	1	6	23	21	22	–
AML M5	X2X3X6	5	1	1	76	3	12	73	+
AML M5	X2X6	1	1	1	3	4	11	87	–
AML M5	X2X3X6	2	2	2	2	5	5	4	+

Table 6. Immunologic phenotype in bone marrow samples without the X2 flag.

cytotype flags		*immunologic phenotype*							
		CD10	*CD19*	*CD20*	*CD2*	*CD3*	*CD5*	*CD7*	*Tdt*
AML M1	X3X6X7	4	2	2	3	9	3	3	–
AML M2	X6	1	20	3	11	15	8	11	–
AML M2	X1X3X5X6	3	3	5	19	15	15	53	–
AML M3	X3X6	3	1	5	29	1	11	7	–
AML M4	X3X6	1	2	1	14	4	3	5	–
AML M4	X6	4	4	2	8	10	8	12	+
AML M4	X3X6	1	1	1	4	5	4	5	+
AML M5	X3X6	2	3	2	10	6	4	10	–

ferences between peripheral and bone marrow blasts, even though the cells showed similar morphological and cytochemical characteristics those observed with the optical method.[19] Of these 16 leukemic bone marrow samples, the X2 flag was observed in 8. In 5 of these latter, the X2 flag could be explained by the presence of blasts having a simil-lymphoid appearance (condensed chromatin, little basophilic cytoplasm, regular nuclear outline) but with the cytochemical and immunological phenotype of myeloid cells. In addition, we found positivity for 2 or more markers of the B and/or T lineage in these 5 cases. In the other 8 cases without the X2 flag, the immunophenotype did not show lymphoid lineage markers.

In conclusion, the VCS system can be used in association with an automated blood counter for better definition of blast cells.[20] Both the quantitative and qualitative sensitivities are good, and allowed a preliminary distinction between ALL and AML. The VCS can discriminate between immature myeloblastic and monocytic forms, on the one hand, and granular and promyelocytic ones on the other. It is the authors' opinion that further studies on VCS bone marrow data could allow a better characterization of acute leukemia.[21]

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