The impact of cell heterogeneity and immunophenotypic changes on monitoring minimal residual disease in acute myeloid leukemia*

T. ŽELEZNÍKOVÁ, O. BABUŠÍKOVÁ

Cancer Research Institute, e-mail: exontzel@savba.sk, Slovak Academy of Science, 833 91 Bratislava, Slovak Republic

Received April 19, 2006

Monitoring of minimal residual disease (MRD) becomes increasingly important for the more accurate stratification of the therapy in acute leukemia. The purpose of this study was to characterize in detail the phenotypes of heterogeneous population in various AML subtypes and to identify the leukemia associated aberrant phenotype (LAP) in individual patients with AML for precise investigation of MRD. The impact of heterogeneity of pathological populations, the effectiveness of location AML blasts on CD45/SSC dot plots in AML patients during follow-up and phenotype changes on MRD monitoring were evaluated in the second step.

Bone marrow samples from 63 patients with AML were analyzed at diagnosis, 33 were selected for monitoring of MRD during follow-up and 13 analyzed at relapse using a wide antibody panel in quadruple combinations by multiparameter flow cytometry.

In 88% of AML patients at least one LAP was defined at diagnosis, two or more aberrancies coexisted in 60% of them. The total number of LAPs identified by application of various combinations of antibodies was 112 (mean = 2.04 LAP/patient) and the median percentage of blasts carrying the LAP was 53.57%. In half of the patients, we were able to detect the presence of at least two subpopulations, which not always shared the same aberrancy. Although AML cells often have light scattering properties similar to those of normal (myeloid and B-lymphoid precursors, basophiles etc.) in a fraction of cases we found also very useful the location on CD45/SSC dot plots for MRD discrimination. In 13 patients relapse occurred and although we found in 69% changes of phenotype when comparing diagnosis and the first relapse, at least one LAP was constant in 92%.

According to our observations, in majority of patients with AML monitoring of MRD by multiparameter flow cytometry is feasible although in some cases could present some specific difficulties owing to their immunophenotypic heterogeneity, similarity with other cell subpopulations or shifts at relapse. In conclusion, investigation of MRD should be based on the phenotypic characteristics of each subpopulation even if it is present in low frequencies.

Key words: acute myeloid leukemia, leukemia-associated phenotypes, AML heterogeneity, antigen shifts, minimal residual disease

Minimal residual disease (MRD) present in bone marrow of patients with AML is responsible for the emergence of relapse. Therefore monitoring of MRD by molecular or immunophenotypic detection methods have crucial value for defining individualized treatment of patients. The application of multiparameter flow cytometry (MFC) for monitoring is based on leukemia-associated aberrant phenotypes (LAPs), i.e. immunophenotypes of leukemic cells that are not or only rarely present in healthy bone marrow and peripheral blood samples [1–3]. Currently the various incidence of LAPs has been reported, depending on the use of large diversity of monoclonal antibodies and their combinations [4–7]. Several studies reported that at least one LAP could be identified in practically every patient with AML, however some with lower sensitivity [8, 9]. The absence of a clear leukemia-associated phenotype is the major limitation to the applicability of flow cytometry to MRD studies. However, this is currently observed in only a minority of patients, and it is likely to become even less frequent as new markers of leukemia are discovered. Furthermore it is known that in AML, the immu-

*This work was partly supported by Grant No.2/4021/6 from Slovak Grant Agency VEGA and grant of project GENOMICS, Subproject No. 9.

nophenotype of leukemic cells may be heterogeneous and several leukemic subpopulations presenting a high diversity of phenotypes may be present at diagnosis. These subpopulations often correspond to different stages of maturation of the pathological clone and one of them may be resistant clone that could be responsible for the relapse of the disease [2]. However, another obstacle for monitoring of MRD except the heterogeneity of AML could be the occurrence of antigen shift during the treatment. Several studies have described shifts in immunophenotype at relapse in AML patients, with different frequencies, probably related to the number of analyzed antigens and their combinations [9, 10–12]. Most of these shifts involve individual antigens, but the leukemic phenotypes generally remain unaltered during the course of the disease [9–11, 13]. The immunological changes are associated with clonal evolution of the disease or else changes related with the treatment of AML. In these cases of secondary leukemia any of the currently available methods for MRD detection would not be efficient [10].

In the current investigation, patients with AML were evaluated at the time of diagnosis and during follow-up by multiparameter flow cytometry. The purpose of the study was to characterize the phenotype of heterogeneous populations in various AML subtypes and to identify the aberrant phenotypes in individual patients with AML for precise investigation of minimal residual disease. As a second step, the evaluation of impact of heterogeneity of pathological populations, the effectiveness of location AML blasts on CD45/SSC dot plots and possible LAP changes on MRD monitoring in AML patients was performed.

Patients, material and methods

Patients and samples. The criteria for entering the study were exact diagnosis of AML based on morphologic, cytochemical criteria of the French-American-British (FAB) cooperative group and also on immunophenotypical criteria.

Overall 63 patients with newly (*de novo* and secondary AML) diagnosed and untreated AML fulfilled these criteria. Thirty three patients were selected for monitoring of MRD, which had criteria of presence of appropriate aberrant phenotype and entering morphological remission. Distribution of patients according to FAB Cooperative Group criteria is shown in Table 1.

The bone marrow (BM) aspirates of patients with AML were collected in heparin anticoagulant tubes at the initial diagnosis and during the follow-up. The results at diagnosis have been stored in electronic form to compare the immunophenotype of bone marrow populations during the treatment and in time of relapse. To define aberrant phenotypes AML samples were compared with those of healthy donors or patients with leukemia (other than AML) or lymphoma undergoing therapy.

Flow cytometric analysis and gating strategy. Multiparameter flow cytometric analysis was done on erythrocyte-lysed whole BM samples using a commercially available lysing solution. The staining procedure has been described in detail previously [14]. After the immunophenotype of suspected AML patients was established, samples were selected and examined by staining with relevant combinations of antibodies for each case. To detect LAPs allowing to monitor MRD and heterogeneity of bone marrow compartment a quadruple marker staining with fluorochromes-labeled MoAbs, fluorescein isothiocyanate [FITC], phycoerythrin [PE], R-phycoerythrin-texas red [ECD] and phycoerythrincyanin 5 [PC5] were used in the following combinations: HLADR/CD13/CD45/CD34; CD11b/CD117/CD45/CD34, CD33/CD7/CD45/CD34, CD4(CD2)/CD56/CD45/CD34, CD14/CD15/CD45/CD13(CD34). For the more detailed analysis of populations in bone marrow, also some other monoclonal antibodies listed in Table 1 were used in altered combinations. All antibodies directly conjugated by fluorochromes were purchased from Immunotech (Marseille, France). MRD studies were performed using the same association of antibodies defining the specific patient profile. Leukemia associated phenotypes were defined by gating on populations displaying an aberrant expression of antigens. Based on our previous experience [14] four different types of aberrant phenotypes were considered: (1) cross-lineage antigen expression, (2) asynchronous antigen expression, (3) over-expression, (4) abnormal light-scatter patterns.

Samples were analyzed within few hours of collection by using multiparameter flow cytometer EPICS ALTRA equipped by Expo 32 program for analysis. For sample at the time of diagnosis, 30,000 events per tube were measured while for the investigation of MRD at least 150,000 events were analyzed for each monoclonal antibody combination.

Results

*Patients.*In the current investigation, 63 unselected patients with AML (11 children and 52 adults) were analyzed between January 2004 and April 2006 at the time of diagnosis by multiparameter flow cytometry applying the panel of monoclonal antibodies described above. The fifty-three patients had de novo AML and ten had secondary AML. The

Table 1. Monoclonal antibodies used in quadruple combinations in AML patients

FITC (clone)	HLADR(Immu-357), CD38(T16), CD2(39C1.5), CD4(13B8.2), CD10 (ALB1),CD11b(BEAR1), CD14(RMO52), CD16(3G8-FITC), CD33(D3HL60-251), CD65(88H7), CD36(FA6.152), CD41(P2),CD42b(SZ2), Glycophorin A (D2.10)
PE (clone)	CD5(BL1a),CD13(SJ1D1), CD15(80H5), CD19 (J4.119), CD34(581),CD56(N901), CD61(SZ21), CD71(YDJ1.2.2), CD117(104D2D1),
PC5 (clone)	CD7(8H8.1), CD33(D3HL60-251), CD34(581), CD45 (J.33)
ECD (clone)	CD45(J.33)

Table 2. Patient characteristics at diagnosis

Gender	Male/female	30/33
Age	Years (median/range)	44/2-83
WBC	x 10^9/l (median/range)	58.6/0,8–369
BM blasts (%)	% (median/range)	65.64/20–95
FAB subtype	AML M0	6 (6p)
	AML M1	12 (10p, 2s)
	AML M2	24 (23p, 1s)
	AML M3	5 (5p)
	AML M4/5	9 (5p, 4s)
	AML M6	1 (1p)
	AML M7	1 (1p)
unclassifiable	AML	3 (3s)

p – primary AML, s – secondary AML

Table 3. Distribution of leukemia associated phenotype in 55 AML patients and their frequencies

LAP expression	patients n=55 N (%)	% pos. AML blasts median (range)
Asynchronous (n=48)		
CD34+CD11b+CD117-	5 (9.1)	48.2 (23–80)
CD34+CD11b+CD117+	8 (14.5)	39.4 (5.9–91)
CD56+CD117+CD34-	1 (1.8)	31
CD56+CD34+CD117-	2 (3.6)	84.5 (83/86)
CD56+CD34+CD117+	6 (10.9)	45.1 (17–99)
CD117+CD65+	3 (5.5)	50.6 (30–70)
CD34+CD15+	2 (3.6)	33 (30/36)
CD33+CD34-CD56+	3 (5.5)	37 (24–53)
CD34+CD33+HLA-DR-CD13-	2 (3.6)	87 (84/90)
CD117+CD33+HLA-DR-CD34-	7 (12.7)	60.7 (18–72)
CD117+CD13+CD33-HLADR-	1 (1.8)	20
CD117+CD33+HLADR+CD34-	3 (5.5)	16.6 (1.8/31.4)
CD4+ CD15+CD14-	4 (7.3)	75.2 (31–100)
HLADR+CD15+CD14-	1 (1.8)	98
Cross-lineage (n=39)		
CD7	23 (41.8)	54.7 (19–97)
CD19	6 (10.9)	20–96 (41.6)
CD2	5 (9.1)	19–90 (38.5)
CD5	2 (3.6)	28/61 (44.5)
CD10	1 (1.8)	20
CD4/CD117	2 (3.6)	35.3 (27/43.5)
Overexpression (n=14)		
HLA-DR++	6 (10.9)	93.7 (65–99)
CD34++	3 (5.5)	60.6 (24–98)
CD33++	1 (1.8)	100
CD13++	1 (1.8)	99
CD117++	3 (5.5)	99.3 (98–100)
Abnormal light scatter (n=11)		
Low FSC/SSC		
CD13	7 (12.7)	60.2 (38–84)
CD33	4 (7.3)	84.3 (73–96)

characteristics of all AML patients at diagnosis are detailed in Table 2.

Heterogeneity of bone marrow populations. The immunophenotypes of heterogeneous populations were examined in bone marrow of all AML patients and compared with healthy donors or patients undergoing therapy. In 28 cases the presence of at least two pathological subpopulations with more or less different antigen expression at diagnosis were detected. The discrimination between the different cell populations was based on: (1) the expression of differentiation-associated antigens (2) differences in light-scatter characteristics with combination of CD45 and (3) the existence of aberrant phenotypes. In 54% of the patients at least one of the subpopulations identified was in minority (<15% of the total leukemic cells). In four patients a LAP was not present at diagnosis. From the rest 24 patients with LAP at diagnosis, in 29% (7 cases) at least one subpopulation with LAP was present; and in 71% (17 cases) all the subpopulations displayed the same (13 cases) or different LAPs (4 cases). The presence of several subpopulations was observed in M4/5 subtype (44.5%) followed by subtype M2 (41.7%) and M3 (40%). More than two subpopulations were observed mainly in M2 subtype, where these subpopulations corresponded to different stages of maturation, starting from the most immature one. High heterogeneity of pathological clones was observed and even the change in the total composition of bone marrow compartments was also found. Furthermore, location of some populations (myeloid and B-cell precursors, basophils, monocytes and in several cases also NK-cells) of bone marrow were seen to merge with location of particular AML blasts on CD45/SSC both at diagnosis and during follow-up (Fig. 1). Therefore the gating on CD45/SSC during follow-up was useful only for exclusion erythroid cells and a proportion of cell debris and hence some other gating strategy based on aberrant phenotype had to be used.

LAP, their frequency and applicability for MRD. In 55 of 63 patients (87.3%) at least one LAP appropriate for MRD monitoring was defined at diagnosis and more than one phenotypic aberration was detected in 33 (60%) of these patients. In 17 (30.9%) patients two independent LAPs, in 6 (10.9%) three LAPs and in 10 (18.2%) four or more LAPs were defined. The total number of LAPs identified by application of various combinations of antibodies was 112 (mean = 2.04 LAP/patient). The inclusion of more than 95% of AML blasts into one LAP was possible only in some patients (16 cases), because in the majority of cases a LAP was not expressed in the whole leukemic population due to phenotypic heterogeneity. Therefore the percentage of AML blasts carrying the LAP was found to range from 1.8% to 100% and hence median LAP expression within the whole pathological population was 53.57%. The most frequent LAP was asynchronous expression, and was mostly observed in M1-M2 subtypes, detected in 38 (69.1%) of 55 AML patients and in majority of these cases at least one progenitor-associated marker (CD34, CD117) was included. Cross lineage infidelity was present in 35 (63.6%) and observed in almost all subtypes, mainly in M0-M2, except M4/5 and M7. The overall frequency of over-expression was observed among all subtypes except M3 and unique M6 and M7. Higher incidence of

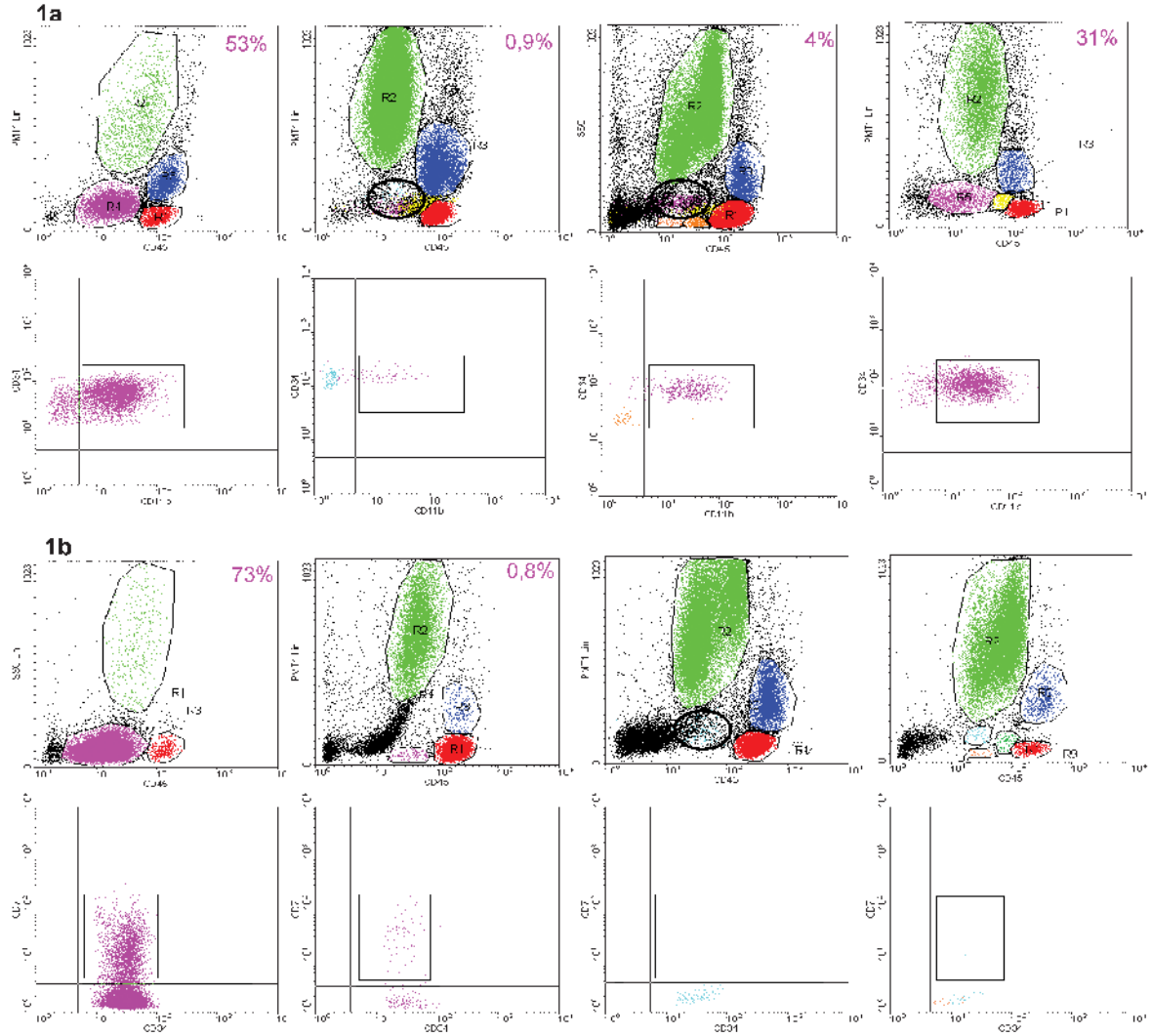

Figure 1. The impact of location of several bone marrow populations on CD45/SSC at diagnosis and during follow-up of gradually relapsing patient (1a) and of patient in remission (1b). The gating strategy on CD34 cells was used for detection of pathological cells with LAP. In the dot plots, red dots depict lymphocytes, blue monocytes, green granulocytes and violet AML blasts. 1a. Example of merging myeloid precursors (turquoise dots) and NK cells (yellow dots) with MRD (violet dots) of bone marrow on CD45/SSC during follow-up. The gating strategy was based exclusively upon one of the aberrant phenotypes (CD34/CD11b) because gating on CD45 could be misleading.
1b. Example of usefulness of location blasts (M0) on CD45/SSC for MRD monitoring. The MRD was defined upon LAP coexpressing CD34/CD7, but dot plot location was also helpful. Although the location of MRD (violet dots) due to low SSC resembled B-cell precursors (orange dots), it differed from the location of myeloid precursors (turquoise dots).

multiple aberrant phenotypes (3 and more) was observed among immature M0 AML cases (67%). The summary and frequencies of LAPs, which have been used for detection of MRD, are given in Table 3.

Stability of LAPs and applicability for MRD. From total 55 patients with LAP at diagnosis in 8 patients the samples were not available for MRD detection due to early death and in 14 due to lack of specimens (samples from 12 patients were able to be evaluated only at diagnosis and the rest two had not entered the morphological remission yet). The remaining 33 patients with LAP appropriate to monitor MRD were included in the further analysis. In our study 83% patients with AML entered morphological remission after one or two courses of chemotherapy induction, but 48.5% (16/33) of these patients had persistent occult disease (MRD ≥ 0.1%). In 12 of them relapse occurred within 3–17 months (median 9 months) and they were analyzed both at diagnosis and at relapse applying a panel of quadruple combinations of monoclonal antibodies. In one patient who relapsed within 17 months the false-negative result of MRD due to phenotypic shifts during follow-up was revealed. In 27 selected patients LAPs were investigated for the applicability

for monitoring of MRD. Although in 9 of 13 patients (69%) some changes in at least one antigen when comparing diagnosis and the first relapse were observed, and at least one LAP was constant in 92% (12/13). The more frequently observed changes were acquisition or loss of antigens expression (8 patients) followed by the changes in the proportion of subpopulations (5 patients). Whereas the ratio for acquisition and loss of individual antigen was balanced, the loss of subpopulation (4 patients) was more common than the gain (1 patient). Changes in myeloid antigens (CD13, CD33, CD14, CD15) and HLA-DR, were the most frequent and were identified in 6 cases (46%) followed by changes in progenitor associated antigens (CD117, CD34) in 3 cases (23%) and changes in lymphoid antigens (CD2) in only one case. Interestingly, there were only slight changes in size (forward scatter) or granularity (side scatter) characteristics, while the location of blasts on CD45/SSC at relapse was stable.

Table 4. Immunophenotype changes of leukemia associated phenotype at relapse, compared to diagnosis

Leukemia associated phenotype (LAPs)		N of patients	N of LAPs at diagnosis	% blasts (median)	N of LAPs at relapse	% blasts (median)
no changes		8	17	62.6 (13-99)	17	54.2 (7–99)
acquisitions		1	1	69	2	82 (69/95)
loss	at least one constant	3	9	58.4 (20-91)	6	69.5 (35–93)
	complete missing	1	1	99	0	0

In 8 patients no change in LAPs at relapse was observed and the loss of LAP was detected in four patients. Three of them had at least one LAP constant between diagnosis and relapse and in one of them only single LAP (over-expression) present at diagnosis disappeared at relapse. In our study we observed only in one case acquisition of a new LAP with 82% of expression. Changes of LAPs and the median frequencies of LAPs between diagnosis and relapse are shown in Table 4.

Discussion

Monitoring of MRD becomes increasingly important in the risk-adapted management of patients with acute myeloid leukemia. The modern methods used at present are dependent on the applicability, specificity and sensitivity of the technique. The applicability of MRD monitoring in AML follow-up is still matter of discussion, because the loss of sensitivity could be suggestible due to immunophenotypic heterogeneity within individual cases and shifts detected at relapse [15]. Therefore we focused our study to the evaluation of impact of heterogeneity of pathological populations, the effectiveness of localization AML blasts on CD45/SSC dot plots and potential LAP changes on MRD monitoring in AML patients.

Our results showed that 87% of patients with AML had at least one aberrant phenotype appropriate for MRD detection and two or more aberrancies coexisted in more than a half of them. The inclusion of all AML cells covered by one LAP was possible only in some patients (29%), while in the majority of cases only a part of the total population of AML was included into one LAP. This was due to phenotypic heterogeneity of AML, which was expressed as a presence of several subpopulations and in most cases also as a wide range of expression intensity of various antigens. It is known, that immunologic heterogeneity not necessarily correspond to morphologic one. In half of all cases we observed the presence of at least two subpopulations with more or less different pathological phenotypic patterns at diagnosis. The frequent presence of several subpopulations in some AML subtypes has also been previously described [4, 10, 16]. The presence of both, the same or different aberrant phenotype on all subpopulations was found in 71% of our patients. The data demonstrate that at least one aberrant phenotype is common to all subpopulations in majority of patients that would allow the simultaneous identification of all subpopulations for monitoring of MRD. In the minority the investigation of MRD should be based on the phenotypic characteristics of each subpopulation. These findings are in accordance with results of MACEDO at al [16], who found that 74% of cell subsets shared the same phenotypic aberrancy.

In this study median percentage of all AML blasts carrying the LAP was 53.57%, while in study of KERN et al [12] was the median lower (25.1%). This fact may be due to the higher expression cut-off levels and different panel of monoclonal antibodies used in our study. Although the median percentage of all AML blasts each carrying LAP was 53.57%, in several cases individual subpopulations expressed different LAPs, so the proportion of AML cells that could be monitor by expression of LAP might be even higher. Therefore it is necessary to apply a broad and comprehensive panel of monoclonal antibodies in diverse antigen combinations to detect all possible LAPs. The proportion of AML patients with identified LAP has substantially increased also by the applications of quadruple combinations with implementation of CD45 for gating strategy [12]. Our findings of usefulness of CD45/SSC are in accordance with results presented by others [12, 17, 18] at the time of diagnosis, but during follow-up it is necessary to use also different gating strategies. Therefore we found the progenitor antigens (CD34, CD117) expressed on the majority of AML [19] also included in majority of LAPs, as very useful for the gating strategy especially for the analysis of MRD. In cases of their negativity, the gating strategy was based upon different aberrant phenotypes presented in Table 1.

We observed that location of particular AML blasts on CD45/SSC may overlap with other cell populations – monocytes, NK-cells and basophils and therefore they may resemble pathologic cells. In these cases the gating on CD45/SSC

could make a false positive results caused by the false identification leukemic myeloblasts and gating exclusively based upon aberrant phenotypes is necessary. We observed that not only monocytes, basophils and NK-cells, but also B-cell precursors and myeloid precursors could occupy common position of leukemic blasts on CD45/SSC dot plots. The precise immunophenotype of B-cell precursors of BM populations had been previously reported [20, 21] and those of myeloid precursors somewhere else [22–24]. We found that location on CD45/SSC together with exact immunophenotype could be of help in MRD monitoring especially by identification of immature blasts (M0) that have much lower light scattering properties than the myeloid precursors. On the other hand, in some of these cases it is important to ensure that immature M0 blasts with immunophenotype $CD34^{+}HLA\text{-}DR^{+}CD38^{+}$ and with coexpression of one B-lymphoid marker are not mistaken as hematogones during follow-up. In such cases it is necessary to examine other B-lymphoid markers. In other subtypes (mainly M1-M2) we found the location of blasts very similar to location of regenerating myeloid precursor. In these cases the discrimination was possible only due to aberrant phenotype. Furthermore we also observed in regenerating bone marrow of non-myeloid leukemia patients myeloid precursors $CD34^{+}$ with different expression of CD13 and CD33 (data not shown). These observations are consistent with those reported by GAIPA et al [22] who found in bone marrow a rare subset of immature myeloid precursors $CD34^{+}CD13^{+}CD33^{-}$; this phenotype should not be considered as aberrant. These observations suggest that the range of normality in myeloid differentiation and aberrant phenotypes need to be established by extensive studies of bone marrows not only from healthy donors but also from patients with non-myeloid leukemia undergoing therapy.

A prerequisite for the validity of MRD monitoring by multiparameter flow cytometry is besides heterogeneity also the stability of LAP between diagnosis and relapse. The shifts (loss or acquisition) in expression of individual markers at relapse may have a relationship to the treatment and they are important for correct assessment of MRD [25]. Although we found in 69% of patients changes in at least one antigen when comparing diagnosis and the first relapse, at least one LAP was constant in 92%. These findings are in accordance with the results presented by BEAR et al [10] and LANGEBRAKE [19] who despite of changes in marker expression between diagnosis and relapse (91% and 88%) found that aberrant phenotypes persisted at relapse in majority of patients. We demonstrated immunophenotype changes in leukemia cells at relapse, compared to diagnosis in 9 of 13 patients, in which at least one LAP was established. Twenty-seven LAPs in 13 patients who relapsed were studied for the impact of LAPs stability and applicability for MRD monitoring in AML patients. In 61.5% of patients, all of the initial 17 LAPs detected at diagnosis remained unaltered at relapse; although, the median percentage of cells caring LAPs could be little different (Tab. 2). In the rest of patients (38.5%) the changes in LAPs were detected. More frequent change observed was loss of LAP (4 patients) while only in one case acquisition of a new LAP was found. Only in one of 4 patients where the loss of LAP was found the false-negative result of MRD due to phenotypic shifts during follow-up was revealed. In the detection of MRD false-negative results have been also described by others and were attributed to phenotypic shifts detected at the time of relapse [10, 11, 26]. The potential adverse effect of this phenomenon is inversely related to the number of marker combinations and revealed LAPs at diagnosis [27]. This implies that preferably at least two LAPs should be used for immunophenotypic MRD monitoring. The changes in proportion of subpopulations also occurred in our study and in two cases only one of subpopulations was observed to be responsible for the relapse of the disease. This was also previously described by MACEDO et al [16] and it is warrant to take into account these findings, and monitoring of MRD should be based on aberrant phenotypes of all subpopulations detected at the diagnosis. In this report monitoring of MRD was realized in 33 patients with AML and only 13 analyzed also in relapse, the frequencies of significant changes of LAPs and especially the results of MRD monitoring could not be estimated accurately yet.

In conclusion to our experience in majority of patients with AML monitoring of MRD by multiparameter flow cytometry is feasible although in some cases could present some specific difficulties owing to their immunophenotypic heterogeneity, similarity with other cell subpopulations or shifts at relapse. This relevance could be decreased by using the extensive and comprehensive panel of monoclonal antibodies in diverse antigen combinations to detect all possible LAPs with carefully evaluations.

The authors acknowledge the physicians from the National Cancer Institute and from the Department of Pediatric Oncology of University Children's Hospital, Bratislava, Slovakia for patient samples and referrals. The authors thank Mrs. A. KOVARÍKOVÁ and Mrs. L. ŠTEVULOVÁ for their excellent technical support.

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