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Correlation of Morphological FAB Classification and Immunophenotyping: Value in Recognition of Morphological, Cytochemical and Immunological Characteristics of Mixed Leukaemias

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Correlation between the FAB classification and immunophenotype was studied in 169 consecutive adult patients with acute leukaemia (AL). The lineage of leukaemic cells could be determined in the majority of cases, whereas 3 patients (1.8%) remained unclassified. In 22 out of 71 patients (31%) with acute myeloid leukaemia (AML) FAB M1 and M2 types, and in 5 out of 16 patients (31%) with chronic myeloid leukaemia (CML) in myeloid blast crisis, leukaemic cells did not express myeloid lineage-related markers, indicating asynchronous expression of cell markers in a substantial proportion of patients. Flow cytometric two-colour immunofluorescence revealed mixed AL immunophenotype in 6 out of 169 patients (3.4%). This group included five CD2$^+$AML (5% of AML tested) and one undifferentiated AL expressing CD10(CALLA), CDw65(VIM-2). The former group included FAB M1, M2, M3 and M4 forms of AML with a single cell population, and an AML M2 patient with both cytochemically and immunologically two separate populations of leukaemic cells. This further illustrates the heterogeneity of the target cell(s) for leukaemogenesis and the level of differentiation of AML cells. However, there was no difference in the treatment response and the remission duration between AML patients and patients with mixed phenotype AML.
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INTRODUCTION

CLASSIFICATION OF acute leukaemias (AL) is based on the morphological–cytochemical, immunological and cytogenetic characteristics of haematopoietic cells [1–3]. This has enabled a rapid and more accurate separation of AL into various subsets, including variants of leukaemias termed mixed, biphenotypic or lineage-promiscuous [3, 4], and a group of leukaemias with asynchronous expression of immunophenotypic and morphological–cytochemical characteristics [5, 6]. In the latter asynchronous group of leukaemias, blasts reveal clear morphological and cytochemical characteristics of myeloid cells but without the expression of myeloid lineage-related immunophenotype, and vice versa [5, 7]. This may be a consequence of pathological maturation of transformed stem cells [1, 6]. This asynchronous group of AL is included in morphologically–cytochemically and/or immunologically unclassified AL [5, 7]. Recognition of the AL subgroups is instrumental for appropriate prognosis and subsequent choice of therapy.

In this study correlation was made among morphological FAB (French–American–British) classification, cytochemical characteristics and immunophenotype of 169 AL. Although similar studies have been performed [5–11], due to great clinical importance of fine subgrouping of AL, our 169 cases are an important contribution to a better understanding of the biology of AL.

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Table 1. Monoclonal antibodies used in this study

Immunophenotype	CD classification
Lymphoid	
Null	HLA-DR, CD34+/−, CD19+
Common	HLA-DR, CD34+/−, CD19+/−, CD10
B	HLA-DR, CD19, CD10+/−, CD20, mIgM, kappa, lambda, CD22
T	CD1, CD2, CD3, CD5, CD7
Myeloid	HLA-DR, CD34+/−, CD33, CD13
Monocytoid	HLA-DR, CD34+/−, CD14, CDw65, CD33, CD13

PATIENTS AND METHODS

Patients

The study included 169 adult patients with AL treated from January 1985 until May 1991 at the Zagreb-Rebro Clinical Center, Zagreb. There were 148 cases of AL diagnosed *de novo*, and 21 patients with chronic myeloid leukemia (CML) in blast crisis. All patients received chemotherapy protocols described previously [12–15]. Complete remission was defined as a reduction in the marrow blast-cell count to less than 5%, with normalisation of peripheral blood cell count and Karnofsky score $\geq$ 90% [16].

Morphology and cytochemistry

The morphological diagnosis of AL was determined in May–Grunwald–Giemsa stained bone marrow and peripheral blood smears. The following cytochemical analyses were also applied: periodic acid–Schiff reagent (PAS), myeloperoxidase (MPO), α-naphthyl-acetate esterase (ANAE), acid phosphatase (AcP) and Sudan black B (SBB) [17–19]. Classification of AL and acute myeloid leukaemia (AML) subtypes followed the FAB criteria modified by Martelsmann and Cheson [3, 20, 21].

Immunophenotyping

Peripheral blood or bone marrow mononuclear cells were separated on a Ficoll–Hypaque gradient, washed twice and used in standard indirect or direct immunofluorescence assay using monoclonal antibodies (Mabs) to cell surface antigens. First-line Mabs included HLA-DR (ia), CD33, CD13, CD19, CD10, CD2, CD5, IgM (Coulter Clone, Coulter, Hialeah, Florida, U.S.A.), CD7 (Behring, Marburg, FRG) and CD34 (BI-3C5) (a generous gift from Dr R. Tindle). Second-line reagents included CD1, CD3, CD4, CD8, CD20, CD22, PLT-1, kappa, lambda (Coulter Clone), CD14, CD22 and Cdw65 (Behring) (Table 1) [7]. Two-colour flow cytometry was done on fresh or cryopreserved cells from 6 patients whose cells expressed both lymphoid and myeloid lineage-associated cell surface markers on standard procedure. For this purpose, a combination of indirect and direct immunofluorescent (IF) procedure or direct two-colour IF procedure were used. Briefly, in the indirect/direct method cells were first incubated with unconjugated Mabs, followed by washing and labelling with fluoroscein isothiocyanate (FITC)-conjugated goat anti-mouse antibody (Coulter). After extensive washing and blocking, the remaining free binding sites on goat anti-mouse immunoglobulin (Ig) by normal mouse Ig, the cells were incubated with phycoerythrin (PE)-conjugated Mabs. In direct/direct method, cells were incubated with a combination of FITC and PE-conjugated Mabs, followed by extensive washing. In all analyses, the matched isotype unconjugated or conjugated controls (Coulter) were used. All samples were immediately analysed on an EPICS-C flow cytometer (Coulter) using standard Coulter software. The fluorescence intensity was measured on 5000 gated cells. Staining of more than 15% of cells above control samples was considered a positive result [7, 22].

Cytogenetic studies

Mononuclear cells from 1–3 ml of bone marrow and/or peripheral blood were prepared following a technique described by Yunis *et al.* [23]. Bone marrow cells were cultured for 24 h, synchronised by methotrexate, and stopped by colcemide 10 min before harvesting. The cell suspension was then treated with hypotonic solution (0.075 mol/l KCl), fixed in methanol:acetic acid 3:1, and washed with a fixative six times. The cells were dropped on a cold wet slide and, after a drying period of 3–8 days, stained by Giemsa solution (pH 6.8) after trypsin pretreatment [23].

RESULTS

The FAB cytomorphological classification and immunophenotypes of 169 consecutive cases of adult AL are shown in Table 2. The data are arranged to document the relationship of the two findings. A disagreement between morphological and immunological classification was seen in 7 out of 169 (4.1%) patients. 18 or 10.7% of cases were unclassifiable by cytomorphological criteria, whereas in 38 out of 169 (22.5%) patients the immunophenotype could not reveal the lineage commitment of the cells. The majority of immunologically unclassified AL were of FAB M1 and M2 types; in 22 out of 71 patients (31%) and in 5 out of 16 patients (31%) with CML in myeloid blast crisis leukaemic cells did not express myeloid-related antigens (CD13, CD33, CD14 or CDw65). In contrast, the majority of morphologically ambiguous or unclassified AL showed a 'common' ALL (CD10, HLA-DR, CD19) immunophenotype. After the correlation of FAB findings and immunophenotype had been accomplished, only 3 out of 169 AL (1.8%) remained unclassified by both methods (Table 2).

In our series of patients, the so-called mixed leukaemic

Table 2. Correlation of FAB-classification and immunophenotype of AL

	Immunophenotype								
	AML			ALL			Mixed	U	
FAB	My	Mo	O	CALLA	T	B	AL	AL	Total
M1	16			2		1	1	6	26
M2	25	2		1			2	16	45
M3	6						1	2	10
M4	6	3					1	1	11
M5	2	5							7
M6								1	1
L1			1	5	8				14
L2	1		3	5	4			3	16
CML-L				4				1	5
CML-M	9			1		1		5	16
U AL			1	9	3	1	1	3	18
TOTAL	65	10	5	27	15	3	6	38	169

U AL, Undifferentiated AL; CML-L, chronic myeloid leukaemia in lymphoblastic transformation; CML-M, chronic myeloid leukaemia in myeloid transformation; My, CD33 and/or CD13 positive antigens; Mo, CD14 and/or CDw65 positive antigens.

Table 3. Immunological characteristics of mixed AL

CD antigens (%)	Patient No. 1 FAB-M1	2 FAB-M2	3 FAB-M3	4 FAB-M4	5 FAB-M0	6 FAB-M2
HLA-DR	62.2	(a) 56.3 (b) 61.7	0	21.1	37.1	38.1
CD34	0	(a) 0 (b) 10.5	0	0	0	37.9
CD33	0	(a) 0 (b) 0	63.1	60.3	0	87.3
CD13	23.4	(a) 21.3 (b) 0	0	0	0	48.0
CD2	89.2	(a) 58.0 (b) 80.5	34.5	21.4	0	91.1
CD10	0	(a) 0 (b) 0	0	0	51.2	0
CDw65	0	0	0	0	92.4	0

CD, Clusters of differentiation antigens according to 4th International Workshop on Human Leukocyte Differentiation Antigens.
(a) and (b) are two different populations in two parametric histograms (FALS × fluorescence).

immunophenotype was found in 6 patients only, representing 3.4% of all patients. The most common antigenic profile seen was co-expression of myeloid-associated antigens (CD13 and/or CD33) and T-lymphocytic antigen CD2, whereas in 1 patient blasts co-expressed both CD10 (CALLA) and myeloid-associated antigen CDw65 (VIM-2) (Table 3). In all but one of these cases, blasts also expressed HLA-DR(Ia) antigen, whereas progenitor cell-associated antigen CD34 was present in 2 patients' cells. In this group of mixed AL, the cytomorphological analysis revealed 2 FAB-M2 cases, 1 FAB-M1, FAB-M3, FAB-M4 and 1 morphologically undifferentiated AL (Table 3). In FAB-M1 type, blasts showed low MPO (1%) and SBB (10%) content. In one FAB-M2 case, blasts had a moderate degree of MPO (30%) and SBB (40%) positivity, while in the other patient they exhibited strong MPO and SBB positivity. In FAB-M4 mixed leukaemia, approximately 20% of blasts contained both MPO and SBB whereas 50% of cells were $ANAE^+$ and this reaction could be inhibited with NaF. Finally, morphologically unclassifiable cases expressed neither of cytochemical marker tested (Table 4).

Leukaemias of mixed phenotype were analysed by means of flow cytometry. In all but 1 patient, the population of leukaemic cells was relatively homogenous with respect to cell size and cell granularity, and was present as a single cell population. However, in 1 patient, a two-parameter histogram revealed two populations of blasts, which differed in the expression of cell surface antigens. One population was HLA-DR, CD13 and CD2 positive whereas the other expressed HLA-DR, CD2 and CD34, with no expression of myeloid-associated antigens (Table 3). Morphologically, this AL was classified as FAB-M2, since it contained a fraction of cells with maturation-related azurophilic granules in the cytoplasm. The blasts were PAS^-, MPO^+ (30%), SBB^+ (40%), and $ANAE^+$ (40%), with no inhibition with NaF. Part of the blasts was morphologically and cytochemically undifferentiated (Table 4).

Table 4. Morphological and cytochemical characteristics of mixed AL

	Patient No. 1	2	3	4	5	6
FAB	M1	M2	M3	M4	M0	M2
MPO (%)	1	30	50	20	0	80
SBB (%)	10	40	50	20	0	80
PAS (%)	0	0	0	0	0	0
ANAE (%)	0	40	0	50*	0	0

* Positive inhibition with NaF.

Clinical characteristics of six mixed phenotype AL are listed in Table 5, illustrated by the duration of the first complete remission (Table 6). In the acute phase of the disease, 4 patients had remarkably high leukocyte counts (above $50 \times 10^9/l$), whereas two of them presented with low leukocyte count. Lymphadenopathy was present in 3 patients, and pathological cytogenetic finding (t 15;17) in 1 out of 4 patients tested. Table 5 also summarises responses to induction chemotherapy and the duration of the first remission. Patients with $CD2^+$AML received AML therapy, while a patient with a $CD2^+$,$CDw65^+$ AL received ALL treatment. 3 patients with $CD2^+$ AML were in remission for more than 10 months, 2 patients relapsed after 3 and 5 months, respectively, whereas 1 patient with $CD10^+$, $CDw65^+$ AL died in the course of therapy (Table 5). By comparing the duration of the first complete remission for all subgroups of AL in our study, the only significant difference was a longer duration of the first remission in the group of patients with a 'common' ALL (Table 6).

DISCUSSION

In our series of 169 patients with acute leukaemia, the breakdown into major immunological subtypes was similar to

Table 5. Clinical characteristics of mixed leukaemias

	Patient No.					
	1	2	3	4	5	6
Age (years)	27	37	47	27	27	63
Leukocytes ($\times 10^9$/l)	188	95	51	54	3.5	1.5
Cytogenetic findings	n	n	nd	n	nd	t(15,17)
Lymphadenopathy	+	+	0	+	0	0
Cerebrospinal fluid	–	–	–	–	–	–
Therapy	AML	AML	AML	AML	AL	AML
Entering remission (days)	21	20	32	29	0*	20
Duration of remission (months)	12	26	3	5	0*	11

n, Normal; nd, not done; *patient died in the course of therapy.

that reported elsewhere [3, 7, 24]. However, several distinct variants with respect to cytology/cytochemistry and immunophenotype of leukaemic cells were seen within non-lymphoid leukaemias in this study.

The majority of our morphologically assessed FAB-M1, FAB-M2 and FAB-M3 AML expressed myeloid-associated (CD1 and/or CD33) or monocyte-related (CD14, CDw65) differentiation antigens, with a stronger tendency toward the former group (Table 2). The FAB-M4 AML similarly separated into the two immunological groups, whereas FAB-M5 type expressed more frequently monocyte-associated antigens; this is generally in good agreement with the results of other authors [24]. However, a fraction of leukaemias in this study remained immunologically unclassifiable with the panel of Mabs used. This observation is consistent with the idea of pathological maturation of leukaemic cells, i.e. earlier asynchronous appearance of the MPO activity than of myeloid antigens in malignant cell of single AL [1, 5, 6]. It is possible that a portion of M3 leukaemias remains immunologically unclassified due to the extended temporal asynchrony of morphological and immunological maturation.

Acute leukaemia expressing differentiation antigens for more than one cell lineage is usually termed biphenotypic, mixed or lineage-promiscuous leukaemia [25–27]. The most common examples include AML expressing some of the lymphoid markers and vice versa. In our study, acute mixed leukaemias were present in only 6 out of 169 adult patients (3.4%). 5 of them were classified as $CD2^+$AML, whereas 1 patient's cells were morphologically undifferentiated and coexpressed CD10 and myeloid-associated antigen CDw65 (Table 3). The latter antigenic combination is rare, and is not encounterd in typical AML [11, 28]. Lymphoid antigen CD10 is rarely associated with other myeloid markers [29, 30]. Leukaemic blasts in 3 out of 5 $CD2^+$AML patients had low MPO and SBB content. This corresponds to the findings of other research groups [1, 31, 32]. On the other hand, one mixed AML FAB-M2 showed MPO and SBB content comparable to that in FAB-M2 type. Mixed AML of FAB-M3 type had 50% MPO and SBB-positive blasts, but this was still lower than usually found in AML-M3. Thus, it appears that cytochemical characteristics of mixed AML vs. AML are not as straightforward as has been suggested [1, 31, 32]. In 1 patient with $CD2^+$AML blasts showed morphological properties of mixed leukaemia, with myeloid and lymphoid markers expressed on different cell populations (third subgroup of mixed AL according to Chesson *et al.*) [3]. One population expressed CD2 and CD13 and the other expressed CD2 and CD34, a haematopoietic progenitor cell marker [24, 33]. The latter population was represented by morphologically undifferentiated MPO and SBB-negative cells, while the former had azurophilic granules in the cytoplasm and contained MPO and SBB (Tables 3, 4).

In comparison to other studies [1, 29, 34], the low percentage of mixed cases in our group of patients could be partly explained by the lack of use of lymphoid markers such as CD7 and TdT, which have a tendency to be coexpressed on AML cells at higher frequencies (approximately 20% of AML cases) [29]. In addition, the reported incidence of $CD2^+$AML varies between 11 and 25% [1, 29], yet in our study this phenotype was found in 5% of AML cases only. Similarly, myeloid antigen-positive ALL was reported in both paediatric and adult groups with approximately equal frequencies, ranging from 11 to 35% of all AL [1, 29, 35]. In contrast, none of the 35 adult patients with ALL in our study expressed myeloid markers (CD13, CD33 or CDw65). It should also be mentioned that in a study of over 500 cases of childhood

Table 6. Duration of the first complete remission in different immunophenotype patterns

Immunophenotype pattern	AML		ALL					
	My	Mo	O	C	T	B	Mix	U
Duration of remission (days)								
Median	180	150	120	390*	240	180	150	180
Range	0–1320	0–300	270–600	30–660	180–6300	120–210	90–780	0–420

My, CD33 and/or CD13 positive antigens; Mo, CD14 and/or CDw65 positive antigens; O, nul ALL; C, calla ALL; T, T ALL; B, B ALL; Mix, mixed leukaemias; U, undifferentiated AL.

* Common (Calla) phenotype leukaemias differed significantly ($P < 0.05$) from those with null and B-phenotype.

leukaemia, Ludwig *et al.* [18] found only 11 cases (2%) of myeloid-positive ALL.

Each case with positive markers of more than one lineage was thoroughly analysed by double labelling and flow cytometry; only leukaemias with a documented 'double-positive' cell population were considered as truly mixed, while the others with ambiguous results were eliminated.

4 out of 6 patients with $CD2^+$ AML had high leukocyte count in peripheral blood and accompanied lymphadenopathy in the acute phase of the disease (Table 5). A similar finding was reported by Cross *et al.* [31] in childhood ALL; however, the clinical behaviour of their patients was unusual, since 5 out of 10 children failed to achieve remission using an AML protocol. The children subsequently responded to an ALL treatment, but overall survival was poor. In our study, all patients with $CD2^+$ ALL achieved remission using an AML treatment with survival rates comparable to that of AML patients. Cerebrospinal fluid of patients with mixed AL did not contain leukaemic blasts (Table 5), which is at variance with data reported by Bail *et al.* [10]. Furthermore, only 1 patient had a cytogenetic defect—t(5; 17), whereas Bail *et al.* [10] found proportionally more cytogenetic alterations in mixed AL than usually observed in patients with AML. There are several reports that AML with certain cytogenetic defects is associated with coexpression of myeloid (CD33,CD13) and CD19 lymphoid antigens [36–38].

Finally, there is abundant literature on the prognostic significance of leukaemia immunophenotype [3, 10, 14, 15, 31, 39, 40], but the consensus opinion is still out of reach. Our data clearly showed that ALL of the so-called common type had longer remission duration than those with null- and B-phenotype (Table 6). This is in agreement with some [41, 42], but not all published reports [43]. The same applies to mixed phenotype AL: some authors ascribed a better prognosis to this phenotype [10], and some ascribe a worse prognosis [31]. Although our number of mixed cases was low, we could not detect any mixed phenotype influence on the remission duration and the outcome of the disease. This is in agreement with some other reports [3, 15, 44].

In conclusion, our study confirmed most of the observations made by others on the relationship between cytomorphology and immunophenotype in acute leukaemia, but also stressed the known fact that the system is highly variable and impossible to delineate fully and at an absolute consensus level. Comparing data from different centres, especially concerning the treatment protocols and the clinical outcome of the disease, might help in better evaluations of mixed leukaemias.

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Expression of CD44 Isoforms Carrying Metastasis-associated Sequences in Newborn and Adult Rats

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Expression of a splice variant of CD44, recognised by the monoclonal antibody (Mab) 1.1ASML, confers metastatic potential to non-metastasising tumour cells (*Cell* 1991, 65, 13–24). To explore whether the metastasis-associated variant of CD44 (CD44v) is expressed under physiological conditions, tissues of newborn and adult rats were stained with the Mab 1.1ASML. The 1.1ASML epitope is, indeed, expressed on the basal layer of the epidermis and the hair follicles as well as on cryptic epithelia in the gut. In addition, ductal epithelia of the pancreatic gland of newborn rats express CD44v. This pattern of expression differs from that of standard lymphocyte CD44 (CD44s). The anti-CD44s mAB Ox50 predominantly stains connective tissue. Although different variants of CD44 may express the epitope recognised by 1.1ASML, cells expressing CD44v share properties with metastasising tumour cells: the stage of proliferation and a restricted degree of mobility. Thus, during metastatic progression tumour cells may reactivate the expression of gene segments which serve highly specialised functions in embryonic and adult tissues.
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INTRODUCTION

THE POLYMORPHIC glycoprotein CD44 [1–4] has been originally described as a homing receptor of lymphocytes [5–7]. Apart from the involvement of CD44 in adhesion of lymphocytes to high endothelial venules, CD44 appears to be involved in lymphocyte maturation and activation [8–10]. Expression has also been found in non-haematopoetic tissues and in a variety of tumour cells [11–16]. While the standard form of CD44 (CD44s) is most abundant on haematopoetic cells, recent reports have shown that tumour cells frequently express larger isoforms of CD44, where additional exons are inserted in the membrane proximal extracellular domain [3, 13, 17–20]. The expression of one class of variants (CD44v), recognised by the Mab 1.1ASML, is causally involved in the formation of tumour metastases [17–19].

The explicit role of CD44v in metastasis formation prompted us to search for its expression in normal tissue. If CD44v was expressed under physiological conditions, the expression pattern, possibly, could provide clues as to the function of CD44v in metastasis formation. Here we show that the epitope for 1.1ASML is expressed in only a very restricted number of normal rat tissues suggesting one or several specialised functions of these CD44 variants.

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