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The Serial Study of c-*myc* Expression in Bone Marrow Biopsy Specimens During Treatment for Acute Myelogenous Leukaemia

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The AMEX method of fixation permitted the serial study of c-*myc* expression in bone-marrow (BM) biopsies obtained from 6 patients with acute myelogenous leukaemia (AML) and one with myelodysplastic syndrome (MDS) during therapy with various cytotoxic and bioactive agents. BM cytotoxic therapy and therapy with bioactive agents was capable of altering c-*myc* expression *in vivo*. While cytotoxic therapy was generally associated with a fall in *myc* expression, it did not produce a dramatic effect on *myc* expression. Recombinant human granulocyte–macrophage colony-stimulating factor (RhGM-CSF) can increase and retinoic acid/α-interferon can decrease c-*myc* expression in myeloid cells *in vivo*.
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INTRODUCTION

THE PROTOONCOGENE c-*myc* is involved in the regulation of both cell proliferation and differentiation [1–7]. Since both of these cellular functions are disordered in acute leukaemia, a large number of studies have been undertaken to identify a possible relationship between the level of c-*myc* expression in leukaemic cells and both the course of the disease and response to therapy [7–19]. Studies of the effects of therapy on c-*myc* expression are of interest since a reduction in c-*myc* expression may be an important aspect of the effects of cytotoxic and bioactive agents.

Serial studies of c-*myc* expression are difficult to perform when peripheral blood or bone marrow aspirates are used since chemotherapy results in a reduction in peripheral blood counts and often produces severe marrow hypocellularity. The availability of a method for evaluating c-*myc* expression in bone marrow biopsies during therapy would obviate these problems [20]. This paper demonstrates that marrow biopsies can be used to study c-*myc* expression during cytotoxic therapy as well as during the administration of bioactive agents, even when the marrow aspirate is too hypocellular to permit study.

PATIENTS AND METHODS

5 patients with acute myelogenous leukaemia (AML) and one patient with myelodysplastic syndrome (MDS) were studied. The clinical and laboratory data for these patients are provided in Table 1. Bone marrow biopsies were obtained at the time of initial presentation and then serially during and after therapy. All patients were studied at the time of initial diagnosis of AML. Patient number 5, who failed to enter remission (CR) after two courses of cytotoxic therapy, was also studied during a 3-day course of retinoic acid/α-interferon and during a third course of cytotoxic therapy. Patient number 4 was studied both at the time of initial therapy and then after relapse. Informed consent was obtained from each patient.

In brief, the treatment protocols listed in Table 1 provide for the following therapies:

(1) AML 89–01: Cytosine arabinoside 2 gm/m^2 twice a day, days 1–6; daunorubicin 30 mg/m^2/day, days 7–9.
(2) AML 88–66: Cytosine arabinoside 100 mg/m^2/day, days 1–10; daunorubicin 50 mg/m^2/day, days 1–3.
(3) AML 88–85: Cytosine arabinoside 2 gm/m^2 twice a day, days 1–6; m-AMSA 100 mg/m^2/day, days 7–9.
(4) AML 90–02: α-interferon (αIFN) 3 × 10^6 u/m^2, days 1 and 3; 13 *cis*-retinoic acid (RA) 75 mg/m^2/day, days 1–3.
(5) AML 90–01: Mitoxantrone 12 mg/m^2/day, days 1–5; VP-16 100 mg/m^2/day, days 1–5.
(6) MDS 90–04: Recombinant granulocyte–macrophage colony stimulating factor (rhGM-CSF) 5 μg/kg/day.

*Fixation, processing and staining for c-*myc *protein*

The AMEX method used to fix the bone marrow (BM) biopsies has been described in detail elsewhere [20, 21] as has the method for staining for c-*myc* protein [21].

The derivation of the anti-*myc* monoclonal antibody *myc* 155, (myeloma clone 155-11c7, Microbiological Associates Inc., Bethesda, Maryland 20816) used in these studies, has also been described previously [22]. As a control for non-specific reactivity with the antibody, the c-*myc* antibody was reacted with the synthetic peptide (residues 171–188) prior to being used to treat the tissue sections.

The entire set of slides from an individual patient was stained at the same time. The slides were deparaffinised in xylene and then in acetone followed by washing (three times) in phosphate buffered saline (PBS). Endogenous peroxidase activity was

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Table 1. Clinical data

Patient no.	Treatment no.	Treatment protocol	Treatment status	Status of disease	Hb (gm/dl)	Tc (×10/l)	Platelets (×10/l)	BM aspiration	Comments on BM
1. 45 years	1	AML 89–01	Pretreatment	New Dx	8.0	1.7	11	BI 38% PM 6%	
Male	2		Day 1		6.9	0.7	7	BI 22% PM 8%	
	3		Day 7		8.1	0.3	13	Dry Tap	Scattered abnormal cells
AML-	4		Day 17		6.5	0.2	24	Dry Tap	Scattered cells, mostly
M1	5		Day 23		7.8	0.2	18	BI 2% PM 54%	progenitors
	6		Day 30	CR	8.2	1.0	33	BI 2% PM 7%	Mostly progenitors
	7		Day 68	CR	9.7	3.3	123		Regenerating marrow
									Marrow in remission
2. 52 years	1	AML 88–66	Day 7	Day 7 of Rx	8.0	1.0	28	BI 8% PM 20%	Extensive medullary necrosis
Male	2		Day 15		8.8	1.1	39	Dry Tap	Stromal injury; very hypocellular
	3		Day 21		9.5	0.7	17	BI 5% mast cells +	Moderately hypocellular
AML-	4		Day 28		8.5	1.1	338	Not interpretable	Insufficient for evaluation
M2	5		Day 35		8.5	3.4	689	BI 4% PM 12%	Hypercellular
	6		Day 54	CR	9.3	6.6	285	N/A	
	7		Post 1st consol.	CR	8.9	5.7	325	BI 3% mast cells +	Normo to hypercellular
3. 36 years	1	AML 88–66	Pretreatment	New Dx	6.9	15.3	162	BI 22% Promono 56%	Markedly hypercellular
Male	2		Day 7		8.2	0.5	93	BI 2% Promono 26%	Stromal injury ++
	3		Day 17		10.3	0.6	32	BI 2% Histiocytes 30%	Stromal injury +++
AML-	4		Day 23		7.6	0.5	16	BI 10% PM 8%	Recovering marrow
M5b	5		Day 30	CR	9.4	7.6	463	BI 4% PM 4%	Recovering marrow
	6		Pre 1st consol.	CR	8.8	7.1	431	BI 2% PM 4%	CR marrow
	7		Pre 2nd consol.	CR	9.4	8.3	203	BI 2% PM 2%	CR marrow
4. 70 years	1	AML 88–66	Pretreatment	New Dx	9.3	130.2	113	BI 2% Promono 85%	Markedly hypercellular
Male	2		Day 7		8.8	0.7	29	Residual Leuk +	Stromal injury ++
	3		Day 17		9.0	0.5	17	BI 1% Histiocytes 44%	Stromal injury ++
AML-	4		Day 25		8.6	0.4	22	BI 9% PM 41%	Regenerating marrow
M5b	5		Day 32	CR	10.4	3.0	427	BI 2% PM 6%	Dyspoietic changes +
	6		Pre 1st consol.	CR	11.6	10.9	121	BI 1% PM 1%	Dyspoietic changes +
5. 45 years	1	AML 88–66	Pretreatment	New Dx	12.6	96.5	90	BI 82%	Moderately hypercellular
Male	2		Day 6		8.7	1.9	23	Residual Blast +; dilute	Mildly hypocellular
	3		Day 26	CR	9.3	11.6	163	BI 5%	Normocellular
AML-	4		Day 35		8.9	22.9	412	BI 0.8%	Mildly hypercellular
M5b	5	AML 88–85*	Day 65	Relapsed	5.5	1.8	27	BI 83%	Hypocellular
	6		Day 45		7.8	0.1	20	Scattered Blast; dilute	Very hypocellular
6. 66 years	1	AML 88–66	Pretreatment	New Dx	9.5	5.8	.078	BI 70% PM 2%	Hypercellular
Female	2		Post–treatment	RD				Very dilute	
AML-	3		Pre 2nd RI		11.2	2.0	181	BI 55%	Normocellular
M2	4		Day 7					BI 55%	Hypocelluar
	5		Day 15					BI 55% PM 5%	Markedly hypocellular
	6	AML 90–02	Pretreatment	RD	10.2	1.4	127	BI 55%	Normocellular
	7	AML 90–01	Day 3/Pre		10.0	0.8	118	BI 47%	Hypocellular
	8		Day 8		9.4	0.5	29	BI 30%	Very hypocellular
	9		Day 22	RD	8.6	0.3	3	BI 56%	Very hypocellular
7. 75 years	1	MDS 90–04	Pretreatment	MDS	9.8	0.4	103	M:E 1:15	Very hypocellular
Male	2		Day 7		8.5	3.1	92	M:E 1:1	Hypo to normocellular
MDS									

quenched using methanol/H_2O_2. The slides were washed in PBS and non-specific binding of the antibody was blocked by applying normal rabbit serum in a dilution of 1 : 10 for 30 min. Excess serum was blotted off and the c-*myc* antibody applied at a concentration of 1 : 100 [10 mg/ml in Tris with 1% bovine serum albumin (BSA)] for 2 h. (Negative controls were prepared by incubating the c-*myc* antibody with 2 mg of the peptide against which it was raised, prior to addition to cells on slides.) Following this, the slides were washed in PBS (3 × 3 min). Finally, the peroxidase–antiperoxidase conjugate was applied at a concentration of 1% for 1 h and then the slides were washed in PBS (3 × 3 min). The colour reaction product was developed using diaminobenzidine (50 mg in 200 ml PBS with 25 ml of 30% H_2O_2) for 7 min. The slides were washed in distilled water and counterstained with Gill's haematoxylin and mounted using fluoromount G.

Evaluation of slides

Consecutive sections of biopsies were stained with haematoxylin and eosin for morphology and with the anti-*myc* antibody or with antibody which had been preincubated with peptide. Each section was assessed independently by two observers (S.D.B and H.D.P) for the staining pattern with regards to the distribution of cells containing c-*myc* protein and the intensity of c-*myc* staining. There was 90% initial agreement between the two observers. The BM was divided into five distinct regions and the

cells within each region assessed for c-*myc* positivity [21]. At least 1000 cells were evaluated (unless the biopsy was very hypocellular). The distribution of cells containing c-*myc* protein was defined as either a diffuse distribution or a "clustered" distribution.

A "cluster" of c-*myc* positive cells was defined as a group of at least three cells containing c-*myc* protein. The level of intensity of staining in individual cells was estimated subjectively by using a three point scoring system: 0 no staining; + minimal positivity; ++ moderate positivity; or +++ heavy staining. To estimate the relative amount of c-*myc* protein present in a marrow biopsy the following equation was utilised: (% + positive cells × 1) + (++ positive cells × 2) + (% +++ positive cells × 3) = estimated c-*myc* protein index. This semi-quantitative estimation of the c-*myc* protein level permitted a comparison of the relative amount of c-*myc* protein in the various biopsy specimens studied.

RESULTS

Serial measurements of c-*myc* expression were made in marrow biopsy specimens obtained from 6 patients, 5 of whom had AML. All 5 patients received remission induction therapy with 4 entering remission. 1 patient with myelodysplastic disease was treated with rhGM-CSF. Table 1 provides demographic data.

A total of 34 marrow biopsies were studied with parallel marrow aspirates evaluated in 33. Of the aspirates, 10 were either moderately or severely hypocellular, 2 were markedly diluted with peripheral blood, and 3 were "dry taps". C-*myc* expression was evaluable in each corresponding biopsy specimen. Hence an evaluation of c-*myc* expression would not have been possible in 1/3 of the patients had BM aspirates been used.

Use of marrow biopsies to evaluate the effects of cytotoxic chemotherapy on myc *expression*

The use of biopsies makes it possible to evaluate the effects of therapy on the proportion of cells containing c-*myc* protein as well as on the geographical distribution of these cells. Figure 1 illustrates the effects of chemotherapy in 1 patient. In this case the diffuse infiltrating pattern of *myc*-containing cells characteristic of florid AML changed during therapy. The proportion of c-*myc* positive cells fell, and at remission c-*myc* containing cells no longer diffusely infiltrated the marrow but rather these cells were organised into small clusters as in normal marrow. A similar pattern was noted in patient number 3.

In patient number 2 the c-*myc* index was increased at the time of remission and the cells containing c-*myc* protein were organised into clusters. In patient number 4, who also entered remission, the dispersed pattern of c-*myc* expression characteristic of florid disease persisted at the time of remission even though the c-*myc* index had decreased. This patient relapsed almost immediately. The marrow of patient number 5, who did not enter remission, showed a fall in the c-*myc* index with the

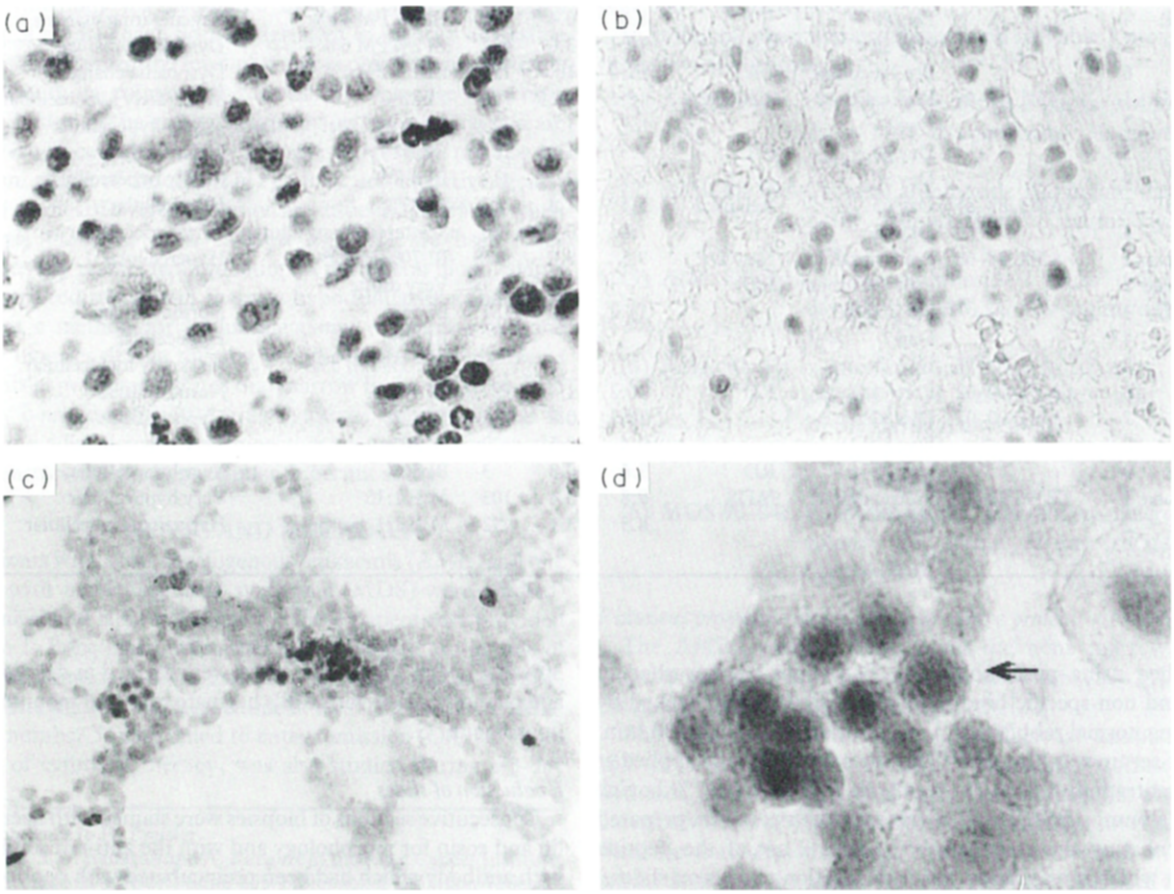

Fig. 1. Serial bone marrow biopsies of patient no. 1: (a) Pretreatment biopsy specimen immunostained with c-*myc* monoclonal antibody and counterstained with haematoxylin showing diffuse infiltration with c-*myc* positive cells (biopsy specimen no. 1; × 40). (b) Day 23 post-treatment biopsy specimen immunostained with c-*myc* monoclonal antibody showing reduction in both absolute number and proportion of c-*myc* positive cells (biopsy specimen no. 5; × 40). (c) A cluster of c-*myc* positive cells seen in the complete remission marrow (biopsy specimen no. 7; × 20). (d) High power view of a cluster of c-*myc* positive cells (biopsy specimen no. 7; × 100).

Table 2. c-myc data derived from bone marrow biopsies

Patient no.	Treatment no.	BM Cellularity	*myc* positivity [%] Total	+	++	+++	*myc* protein index	Localisation	Comments
1	1	Hypercellular	90	31	34	25	174	Dispersed positivity	
	2	Normo cellular	77	22	27	28	160	Dispersed positivity	Paratrabecullar positivity +
	3	Hypocellular	32	30	2	—	34	Dispersed positivity	No paratrabecullar positivity
	4	Hypocellular	60	32	28	—	88	Dispersed positivity	More cellular areas more positive for c-*myc*
	5	Normo to hypercellular	29	20	7	2	40	Dispersed positivity	
	6	Hypercellular	14	13	1	—	15	clonal positivity	Non-specific positivity; regenerating marrow
	7	Normo to hypercellular	12	5	3	4	23	Clonal positivity	Non-specific positivity seen; regenerating marrow
2	1	Normo to hypocellular	20	13	6	1	28	Dispersed positivity	Stromal injury +
	2	Hypocellular	3	3	—	—	3	Dispersed positivity	Stromal injury + +
	3	Hypocellular	22	14	4	3	31	Dispersed positivity	Stromal injury +
	4	Only bone	—	—	—	—	—	—	Only bone
	5	Normocellular	11	10	1	—	12	Dispersed positivity	Stromal injury +
	6	Hypercellular	25	17	6	2	35	Clonal positivity	Non-specific positivity seen
	7	Hypocellular	4	3	1	—	5	Clonal positivity	Non-specific positivity seen
3	1	Very hypercellular	35	34	1	—	36	Dispersed positivity	No clonal positivity seen
	2	Hypocellular	42	38	2	2	48	Dispersed positivity	Areas of hemorrage seen
	3	Very hypocellular	Very hypocellular. Hardly any cells seen						
	4	Hypocellular	55	43	11	1	68	Dispersed positivity	Some islands of regeneration More positive for c-*myc*
	5	Normocellular	66	48	14	4	88	Dispersed positivity	Areas of hemorrage seen
	6	Normocellular	64	40	17	7	95	No clonal positivity	Areas of regen. strongly positive
	7	Normocellular	5	4	1	—	6	Clonal positivity	No non-specific positivity seen
4	1	Very hypercellular	69	48	17	4	94	Dispersed positivity	No clonal positivity seen
	2	Very hypocellular	Only bone fragments and blood clot: no cells seen						Haemmorage +
	3	Very hypocellular	66	50	14	2	84		Haemorrage +
	4	Very hypocellular	47	43	4	—	51	Dispersed positivity	Haemorrage +
	5	Hypercellular							
	6	Normocellular	16	5	8	3	30	Clonal positivity	Non-specific positivity seen
5	1	Hypercellular	60	23	26	11	108	Dispersed positivity	Paratrabecullar positivity + +
	2	Normocellular	65	50	12	3	83	Dispersed positivity	Stromal injury +
	3	Very hypercellular	34	22	13	—	46	Dispersed positivity	Paratrabecullar positivity
	4	Normocellular	1	1	—	—	1	Occ. cell positivity	Lots of non-specific positivity
	5	Very hypocellular	30	27	3	—	33	Dispersed positivity	Paratrabecullar positivity seen
	6	Extremely hypocellular	27	27	—	—	27	Dispersed positivity	Stromal injury + +
6	1	Hypercellular	56	43	11	2	71	Dispersed positivity	
	2	Hypocellular	37	27	8	2	49	Dispersed positivity	Stromal injury +
	3	Hypocellular	68	40	22	6	102	Dispersed positivity	Lot of cells are normal in regeneration marrow elements
	4	Extremely hypocellular	26	26	—	—	26	Dispersed positivity	Non-specific positivity seen; stromal injury
	5	Very hypocellular	51	36	8	5	69	Scattered positivity	Stromal injury + +
	6	Hypocellular	40	30	10	—	50	Dispersed positivity	Some islands of regeneration. Show strong positivity
	7	Hypocellular	16	15	1	—	17	Scattered positivity	Stromal injury +
	8	Very hypocellular	45	33	10	2	59	Dispersed positivity	Stromal injury + +
	9	Very hypocellular	41	12	21	8	78	Dispersed positivity	Stromal injury + +
7	1	Very hypocellular	47	36	9	2	50	Dispersed positivity	
	2	Hypo to normocellular	61	32	17	12	102	Dispersed positivity	More positivity around sinusoids.

persistence of a diffuse infiltration pattern of c-*myc* containing cells.

Tables 1 and 2 provide the numerical data.

Use of marrow biopsies to evaluate the effects of bioactive agents

Patient number 5 failed to enter remission despite having received two courses of intensive remission induction therapy. The patient then received a 3-day course of retinoic acid/α-interferon. C-*myc* expression, which had been stable on two prior measurements, dramatically fell in terms of both the per cent of positive cells and the c-*myc* index. When measured 8 days later, while the patient was receiving a third course of cytotoxic therapy, c-*myc* expression had returned to preretinoic acid/α-interferon levels.

Patient number 6 received rhGM-CSF as treatment for myelodysplastic syndrome. Prior to treatment the patient marrow was severely hypocellular with few cells containing c-*myc* protein. After 2 weeks of rhGM-CSF therapy, the marrrow was cellular with many c-*myc* containing cells. During this time the patient's white blood cell count rose from 0.4×10^9/l to 3.1×10^9/l.

DISCUSSION

The pretherapy levels of protooncogene expression provide important prognostic information [7, 11, 15]. It is possible that therapy itself may alter the level of expression of these genes thereby altering cell behaviour. If this were the case, then measurement of protooncogene expression during therapy could provide additional prognostic information. Additionally, since bioactive agents are administered with the hope of altering cell behaviour, the ability to assess the effects of these therapies on gene expression should increase our ability to understand the effects of these agents. In many situations, however, it is not possible to make these assessments since therapy may be associated with a fall in peripheral blood counts and with the production of marrow hypocellularity.

The study described here demonstrates that for c-*myc* expression it is now possible to make serial measurements throughout therapy, even in the face of a fall in blood counts and marrow cellularity. The method for assessing c-*myc* expression described here has several additional advantages over the use of marrow aspirates. The first is that the use of biopsy specimens makes it possible to evaluate the physical distribution of cells containing c-*myc* protein. Secondly, the cell populations obtained by BM aspiration may not be reflective of the true cell population of the BM because aspirates are invariably diluted by peripheral blood and because all marrow cells may not be equally aspiritable [23]. The use of marrow biopsies avoids these potential problems [24].

While the study described here is in its infancy, some interesting initial observations should be noted. During cytotoxic chemotherapy dramatic changes in c-*myc* expression did not occur. Rather, expression fell gradually, if at all, during chemotherapy and in the 2 weeks thereafter. It is of interest that in the initial remission BM of some patients c-*myc* expression returned to the "normal cluster" pattern [21] while in others, who also entered CR, a "dispersed" pattern similar to that present during active leukaemia persisted. The persistence of a dispersed pattern of c-*myc* expression may merely reflect the rapid proliferation of a normal recovering marrow or may indicate that normal haematopoiesis has not resumed.

Of special interest are the observations made during the treatment of patient number 5. While in this patient two different types of intensive cytotoxic chemotherapy produced no change in c-*myc* expression, 3 days of retinoic acid/α-interferon administration was associated with a dramatic reduction in c-*myc* expression. In studies described elsewhere we have reported that a significant slowing of leukaemic cell proliferation can occur when this combination of bioactive agents is administered to patients with AML [25]. The fall in c-*myc* expression and the slowing of leukaemia cell proliferation may be related. Given that the regrowth of leukaemia cells between courses of cytotoxic therapy is a common cause of treatment failure [25–27], the effects of retinoic acid/α-interferon on c-*myc* expression and on cell proliferation may provide a means for increasing the efficacy of cytotoxic therapy by slowing the regrowth of leukaemia cells between courses of therapy.

The study described here demonstrates the feasibility of serially studying c-*myc* expression in the leukaemia cells of AML patients receiving cytotoxic or bioactive therapy. The ability to make these measurements provides an opportunity for conducting studies which were hitherto not possible to conduct.

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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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