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Parallel Studies of Clonogenic Leukaemia Cells and the Leukaemia Cell Population as a Whole in Acute Myelogenous Leukaemia

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The clonogenic cells in patients with acute myelogenous leukaemia (AML) were evaluated with respect to the relationship between primary and secondary cloning capacity and the proliferative and molecular biological characteristics of the leukaemia cell population as a whole. Secondary cloning capacity was correlated with primary cloning efficiency, and with the ability of the clonogenic cells to produce large sized clones. The cloning capacity of AML cells was unrelated to the cell cycle characteristics of the leukaemia cell population *in vivo* or to the level of myc, myb, fms, or interleukin (IL)1β expression. The sensitivities of the clonogenic cells to cytosine arabinoside and daunorubicin were inversely correlated with the ability of the leukaemia cells to produce large sized clones *in vitro*. This latter observation may explain the reported relationships between the clonogenic capacity of AML cells and response to chemotherapy.

Key words: acute myeloid leukaemia, clonogenicity, primary cloning efficiency, secondary cloning efficiency, cell cycle characteristics, drug sensitivities, MYC, MYB
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INTRODUCTION

HUMAN ACUTE myelocytic leukaemia (AML) cells, capable of clonogenic growth *in vitro*, represent a sub-population of cells whose properties appear to differ from that of the leukaemia cell population as a whole. The clonogenic cells have a more immature immunophenotype than that of the population as a whole [1], and the clonogenic subpopulation has a higher proportion of cells in S-phase than the leukaemia population as a whole [2]. While all clonogenic cells are CD34+, not all CD34+ AML cells are clonogenic *in vitro* [3, 4]. Additionally, the demonstration that some of the clonogenic AML cells have the capacity to 'self renew' suggests that, even within the clonogenic cell population, there are sub-populations with different proliferative capacities [5, 6].

The study described here was conducted to investigate the possible relationships between the characteristics of the leukaemia cell populations as a whole and the clonogenic cells.

PATIENTS AND METHODS

Patients and preparation of cells

This study consisted of specimens obtained from 43 patients with AML. Informed consent was obtained from each patient. Forty-seven bone-marrow (BM) and 46 peripheral blood (PB)

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specimens were obtained from these patients and subjected to Ficoll–Hypaque (d = 1.077g/cm^3; Sigma, St Louis, Missouri, U.S.A.) density cut centrifugation. The low-density cells were collected, washed in phosphate-buffered saline (PBS), and resuspended in RPMI-1640 medium (Gibco, Grand Island, New York, U.S.A.).

Semisolid cultures of leukaemia colony-forming cells (CFC) to determine primary cloning efficiency (PE1)

Cells were suspended at 10^6 cells/ml in RPMI-1640 medium supplemented with 10% fetal calf serum (FCS) alone or in medium to which various growth factors were added. Recombinant human granulocyte–macrophage colony-stimulating factor (rhGM-CSF) (Genetics Institute, Cambridge, Massachusetts, U.S.A.), interleukin 3 (IL-3) (Genetics Institute) or phytohaemagglutinin-leucocyte-conditioned media (prepared in our laboratory) were added to the medium at concentrations of 100 μ/ml, 100 μ/ml and 10% v/v, respectively. The cells were placed in methylcellulose semisolid cultures.

The CFC assays were performed in triplicate. 2×10^5 cells/ml were plated in 1 ml of Isocove's modified Dulbecco's medium (IMDM; Gibco) supplemented with 25% FCS and 0.9% methylcellulose in 35-mm tissue dishes. Cultures were maintained in an incubator at 37°C in 5% CO_2—balance room air at 100% humidity for 14 days. Clones consisting of <10 cells, ≥10 cells, <20 cells and ≥20 cells were scored at day 14. PE1 was calculated as:

$$\frac{\text{no. of CFU scored on day 14}}{\text{no. of cells plated}} \times 100$$

Secondary cloning efficiency (PE2) assays

The cells in the primary cultures were harvested on day 14 and resuspended, mixed and pooled. They were washed, counted and their viability assessed by Trypan blue dye (Gibco) exclusion. Cytospin slides, stained with Wright–Giemsa stain, were used for morphological analysis. Slides were also fixed in formaldehyde-buffered saline for myc protein studies. 2×10^5 cells/ml were replated in methylcellulose and clonal growth assessed as described above in primary cloning cultures in the presence of rhGM-CSF (100 μ/ml). Cultures were evaluated at 14 days. PE2 was calculated as:

$$\frac{\text{no. of CFU scored on day 14 after replating}}{\text{no. of cells plated}} \times 100$$

Estimation of [methyl-3H]thymidine ([^{3}H]TdR) suicide index (Si) [2] and sensitivity to cytosine arabinoside and daunorubicin

The cells were suspended in IMDM medium supplemented with 10% FCS, at 5×10^6 cells/ml, and incubated for 1 h at 37°C in a humidified atmosphere containing 5% CO_2. [^{3}H]TdR (Amersham, U.K.) was then added to the cells at a final concentration of 40 μCi/ml. The incubation was continued for another hour, after which the cells were washed three times with IMDM containing cold thymidine at a concentration of 100 μg/ml of medium. Following this, a cell count and the Trypan blue dye exclusion test for viability was performed, and the cells plated at a concentration of 2×10^5 live cells/ml in methylcellulose with rhGM-CSF (100 U/ml). Marrow or PB cells, which were not exposed to ^{3}[H]TdR, were incubated and washed in an identical manner, and served as controls. The suicide index was calculated as:

$$\frac{\text{no. of control colonies} - \text{no. of colonies produced by cells incubated with } ^3\text{[H]TdR}}{\text{no. of control colonies}}$$

To assess sensitivity to cytosine arabinoside (araC) and daunorubicin (DNR), araC at 0.3 μg/ml and DNR at 0.3 μg/ml were substituted for the ^{3}[H]TdR [7].

Cell cycle measurements of the leukaemia cell population as a whole in vivo

Estimation of percentage of S-phase cells [8]. BrdU was administered intravenously to 16 patients at a dose of 100 mg/m^2 in 50 ml of 0.9% normal saline for 1 h using a constant rate infusion pump. At the end of the infusion, PB, bone marrow aspirate (BMasp) and biopsy samples were obtained. Five millilitres of BM were aspirated into a plastic syringe containing 2 ml of 6% sodium citrate. The specimen was layered on Ficol–Hypaque as described above. The low density cells were recovered, washed in RPMI 1640 cell culture medium and resuspended at a concentration of 5×10^6 cells/ml in RPMI 1640 supplemented with 10% FCS. All processing was conducted in the cold.

The biopsy samples obtained at the end of BrdU infusion were fixed in Bouin's solution and processed in glycolmethacrylate (GMA) [8], mounted on coverslips and treated with the monoclonal anti-BrdU antibody, as described before [8]. After processing, the coverslips were counterstained with haematoxylin and May–Grunwald stains. At least 200 cells were counted in each biopsy section, in at least five different fields, by a single observer.

Determination of duration of S-phase (T_s) and the total cell cycle (T_c)

The post-BrdU BM aspirate was labelled with tritiated thymidine ([^{3}H] TdR) as described in detail elsewhere [8]. T_s was calculated by the formula as developed by Wimber and Quastler [9], and the T_c by the formula: $T_c = T_s \times GF/LI$, where GF is the growth fraction and LI is the percentage of S-phase cells obtained from plastic-embedded biopsies [8]. Since we were calculating the doubling times of only those cells actively engaged in cycle, the GF was assumed to be 100% in each case.

Measurement of myc and myb expression in AML cells prior to assessment of their clonogenic potential [10]

The low density cells described above were washed and resuspended in 1% paraformaldehyde for 30 min at 4°C. These fixed cells were permeabilised by resuspension in IFA (10 nM Hepes, 150 nM NaCl, 4% FCS, 0.1% sodium azide in distilled water) with Triton-X 100 (Sigma) for 5 min, pelleted and resuspended in 100 μl of IFA with Triton-X100. For myc staining, the cells were incubated with a mouse monoclonal antibody (MAb) directed against myc peptide (Microbiological Associates, Bethesda, Maryland, U.S.A.) at a concentration of 1 μg per 1×10^6 cells for 30 min at 4°C. A negative control was included in which the myc antibody had been neutralised by pre-incubation with a 2-fold greater concentration (by weight) of the myc peptide immunogen for 1 h at room temperature. For myb staining, a mouse MAb against a myb peptide (Microbiological Associates) was used at a concentration of 1 μg per 1×10^6 cells for 30 min at 4°C. For a negative control, the myb antibody was neutralised by pre-incubation with a 2-fold greater concentration (by weight) of the myb peptide immunogen for 1 h at room temperature. The cells were then

washed twice with IFA with Triton-X 100, resuspended in 100 μl of the same buffer and incubated with fluoroscein isothiocyanate (FITC)-conjugated goat-anti-mouse IgG (TAGO Inc.) at a concentration of 3.5 μg per 1×10^6 cells in the dark for 30 min at 4°C. After another wash in IFA, the cells were resuspended in 0.5 ml of PBS and analysed by flow cytometry.

Flow cytometric analysis was performed using a FACscan (Becton Dickinson, San Jose, California, U.S.A.). Instrument settings were adjusted using CaliBRITE beads (Becton Dickinson). Forward light scatter and 90° scatter were set to include all lymphocytes, granulocytes and the immature cell population.

The photomultiplier tube No. 1 (FL-1 PMT) was optimised using the negative control. The FITC-associated green fluorescence was collected, amplified logarithmically and stored in the LIST mode. Ten thousand events were collected from each tube and later analysed using the Consort-30 program (Becton Dickinson). Histogrammes created from the green signals of the negative control were used to set a marker so that more than 99% of the negative population fell to the left of the marker. The marker was used to determine the percentage of positivity in the population, and the shift in peak mean channel of fluorescence was determined after eliminating the marker.

Assessment of gene expression by northern blot analysis

RNA was prepared from AML cells by centrifugation through 5.8 M caesium chloride. Five micrograms of whole cell RNA were size separated in 1.2% agarose gels, transferred to nylon mesh paper (Xeta Bind; Meriden, Connecticut, U.S.A.) and hybridised with a random primer labelled probe. These methods and the sources of the probes are described in detail elsewhere [11, 12].

Northern blots were simultaneously hybridised with probes for IL-1β and myb. After autoradiography, the blots were rehybridised with a probe for β actin. The RNA band densities for myc, myb, fms and IL1β were measured on the autoradiographic film, and were normalised by division of the autoradiographic densities of these bands by the β-actin mRNA density in the same lane. These densities were used to rank the level of expression of each gene for each RNA specimen relative to the other specimens [11].

Measurement of myc expression in cells obtained after primary cloning

Myc expression was also measured in cells after primary cloning. The cytospin slides prepared from cells after primary cloning were fixed by dipping into buffered formaldehyde fixative (Baxter, Illinois, U.S.A.) for 30 min. The slides were washed and endogenous peroxidase activity quenched by placing the slides in a bath containing 190 ml methanol and 3 ml 30% H_2O_2 (15 min). After washing, non-specific binding of the antibody was blocked with normal rabbit serum at a dilution of 1:10 (30 min). The myc antibody (same antibody as used in the flow cytometry studies described above) was added at a concentration of 1:100 [10 μg/ml in Tris with 1% bovine serum albumin (BSA)] to the coverslip and incubated for 2 h (negative controls were prepared by pre-incubating the myc antibody the peptide immunogen). The slides were then washed in PBS containing 3, 2 and 1% normal human serum (NHS) for 10, 5 and 5 min, respectively. The secondary antibody (rabbit immunoglobulin to mouse immunoglobulin) was used at a concentration of 4% in Tris containing 5% NHS and 1% BSA. The incubation lasted for 1 h, the slides were washed and the peroxidase–anti-peroxidase conjugate applied at a concentration of 1% for 1 h. After washing, the colour reaction product was developed using di-amino-benzidine (50 mg in 200 ml PBS with 25 μl 30% H_2O_2). The slides were washed once more, counterstained with Gill's haematoxylin, and mounted using fluromount G.

When positive, the myc staining was localised in the cell nucleus. For evaluation of myc positivity, 200 cells were counted and then the percentage of myc positivity determined. Control slides showed no positivity.

Myc expression in individual colonies was also assessed in some primary cultures. To this end, buffered formaldehyde fixative was added directly to the Petri dish and the entire dish processed for c-myc staining using the peroxidase anti-peroxidase (PAP) technique described above.

Statistical methods

The Spearman rank test was used to determine the correlation coefficients and the associated P values.

The paired t-test procedure, using direct numbers (for PE2 studies) or using square-root transformed data (for PE1 studies), was used to determine if the differences in total numbers of colonies produced by different cell populations and subpopulations were statistically significant. As noted above, the level of gene expression as assessed by northern blot analysis was a rank value [11]. These ranks were used in the analysis.

RESULTS

Studies of the relationship of secondary clonogenic capacity to proliferative characteristics and to myc expression during primary cloning

There was an intention to assess the secondary cloning capacity of the leukaemia cells of 51 primary cultures (BM and PB specimens were cultured from 8 patients). However, in 30/51, too few cells ($<0.1 \times 10^6$) were recovered from the primary cultures to permit assessment of PE2. PE2 was assessed in 21 primary cultures containing rhGM-CSF, and in 18 controls in which rhGM-CSF was absent. Only four control cultures manifested secondary cloning capacity, while secondary cloning was produced by 11/20 cultures containing rhGM-CSF. Considering the control cultures, there was a tendency for the PE1 of those which manifested secondary cloning capacity to be greater than that for cultures which did not (Figure 1a). The same was true for primary cultures established in the presence of rhGM-CSF. For these specimens, those that did not manifest secondary cloning capacity, the PE1 was 0.05 ± 0.04 while the PE1 for cultures which had secondary cloning capacity the PE1 was 0.17 ± 0.03 ($P = 0.005$; Figure 1b). The PE1 and PE2, for these cultures were significantly correlated ($r = 0.7052$; $P = 0.0005$; Figure 1c).

A relationship was sought between the size distribution of the clones produced during the initial culture period and secondary cloning efficiency. Significant correlations were found between the absolute number of colonies consisting of >10 cells and those consisting of >20 cells and PE2, with r values of 0.6806 ($P = 0.003$) and 0.6514 ($P = 0.005$), respectively. When the relationship between the percentage of colonies consisting of >10 cells and those consisting of >20 cells were related to PE2, similar correlations were found.

An attempt was made to relate secondary clonogenic capacity to the characteristics of the leukaemia cell population as a whole during the initial period of cloning. Secondary cloning capacity was unrelated either to the number of cells recovered from the

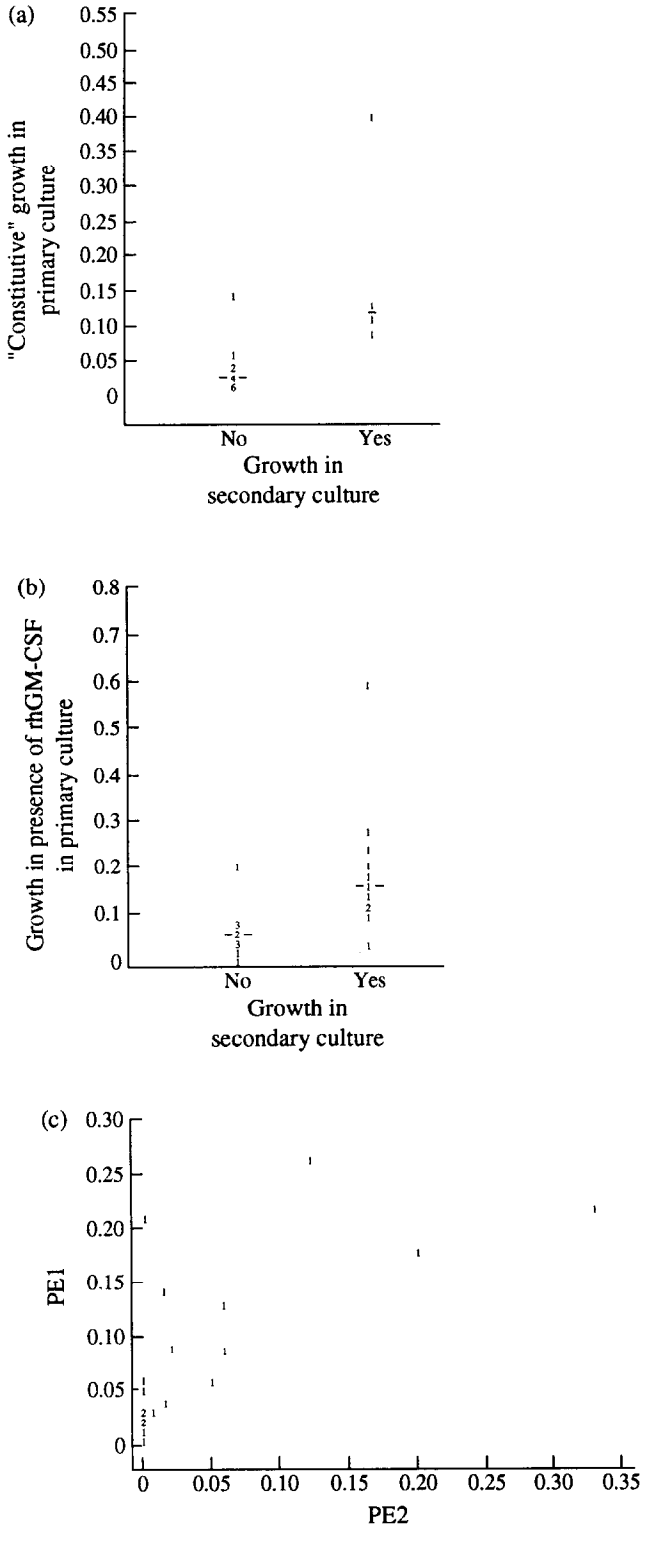

Figure 1. (a) Relationship between constitutive clonogenic growth (PE1) and secondary cloning capacity. (b) Relationship between clonogenicity in the presence or rhGM-CSF (PE1) and secondary cloning capacity. (c) Correlation between PE1 and PE2 of cells plated in the presence of recombinant human granulocyte–macrophage colony-stimulating factor.

methylcellulose after primary culture (which contained rhGM-CSF) or to the proportion of immature cells present. The recovered cell number per plate for those primary cultures which failed to produce secondary colonies was $0.85 \pm 0.27 \times 10^5$ with 0% blast cells. The corresponding values for cultures which produced secondary colonies were $0.67 \pm 0.15 \times 10^5$ and $16 \pm 22\%$, respectively. Hence, while the absence of blast of cells at the end of primary culture was associated with a PE2 of zero, the presence of blast cells was not invariably associated with secondary cloning capacity.

Myc expression was assessed *in situ* in colonies in the methylcellulose plates. Figure 2 illustrates two of the patterns of myc expression which were detected within colonies. In some colonies, all cells contained myc protein, in others, none contained the protein, while in still other colonies, some cells contained the protein while others did not. In all cases, myc expression was restricted to the nucleus. Since it was not practical to evaluate a sufficient number of colonies to attempt to relate myc expression to PE2, myc expression was assessed in the cells recovered after primary culture prior to secondary plating. No relationship between myc expression and PE2 was found. Of interest is the observation that myc expression was not restricted to immature myeloid cells, but was also present in the nuclei of some morphologically mature macrophages (Figure 2).

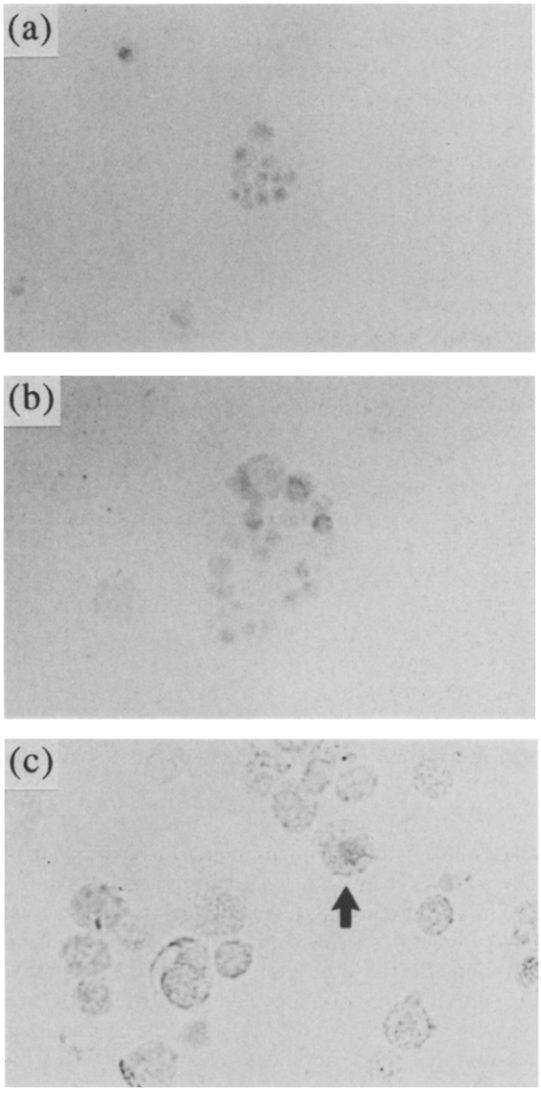

Figure 2. Myc expression during primary culture. (a) Myc protein present in every cell in the colony. (b) Myc protein present in some cells and not in others within an individual colony. (c) Arrow indicates myc protein within the nucleus of a mature macrophage.

Relationship of the sensitivity of clonogenic cells to araC, DNR and tritiated thymidine [^{3}H]TdR to clonogenic capacity

Relationships were sought among the proliferative characteristics of the clonogenic cells and the sensitivity of the clonogenic cells to each agent. For araC and DNR, there was a suggestion of an inverse relationship between PE1 and drug sensitivity, with r values of -0.4523 ($P = 0.09$) and -0.4089 ($P = 0.17$), respectively. For [^{3}H]TdR, the r value was -0.09. Statistically significant negative correlations were found between the number of colonies consisting of ≥20 cells and the percentage of clonogenic cells killed by araC ($r = -0.5622$, $P = 0.03$) or by DNR ($r = -0.7066$, $P = 0.01$). No relationship between colony size and sensitivity to [^{3}H]TdR was detected nor was any relationship between PE2 and sensitivity to araC, DNR or [^{3}H]TdR found.

Studies of the relationship between the characteristics of the leukaemia cell population as a whole and the characteristics of the clonogenic cells

French–American–British (FAB) type. With respect to morphology of the leukaemia cell population, no relationship between FAB type and clonogenic characteristics was discernible.

Cell cycle characteristics. In vivo cell cycle measurements were performed in 16 patients whose cells were cloned *in vitro* (Table 1). No relationships were detected between the proportion of cells in S-phase, the duration of S-phase or the duration of the cell cycle and whether or not the leukaemia cells were clonogenic *in vitro*. Further, no relationships were discernible between these cell cycle characteristics and cloning efficiency or size distribution of the colonies produced.

Protoncogene expression. The proportion of cells expressing the myc gene and the myb gene at protein level were measured in 61 and 44 specimens, respectively, prior to assessment of the clonogenic capacity of the leukaemia cells. Figure 3 shows the distribution of myc and myb expression in specimens which did or did not clone *in vitro*. No relationships were found. Among the specimens with preculture myc and myb measurements, 11 and 7, respectively, were studied for secondary clonogenic capacity. No relationships were discernible.

Northern blot analysis of gene expression at the RNA level were performed in 21 specimens obtained prior to the clonogenicity studies. The levels of expression of myc, myb, fms and IL1β were compared for specimens which did or did not produce clonal growth *in vitro* in the presence or absence of rhGM-CSF. No differences were noted. Relationships were also sought between the level of gene expression and cloning efficiency per

Table 1. Cell cycle data

	% S* BM aspirate	% S* BM biopsy	ts† (h)	tc‡ (h)
n	16	16	16	16
Mean	9.0	26.0	17.0	68.6
S.D.	5.8	8.1	8.4	31.3
Median	8.2	24.3	15.2	69.0
Minimum	1.2	15.4	4.2	14.9
Maximum	21.5	49.7	34.8	111.1

* % cells in S-phase. † Duration of S-phase. ‡ Duration of total cell cycle.

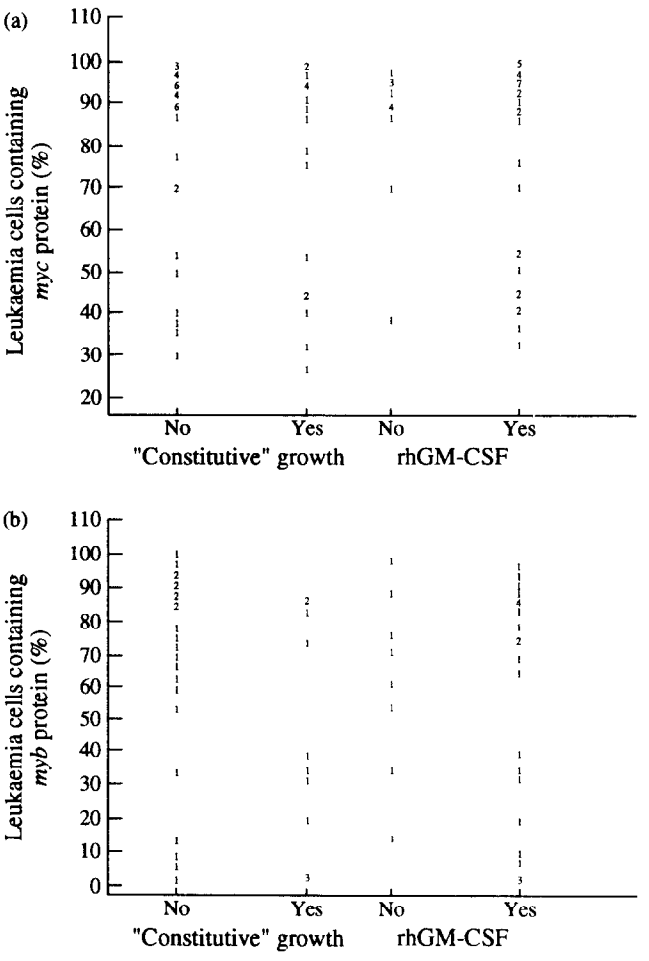

Figure 3. Relationship between protooncogene expression and clonogenic growth. (a) myc expression. (b) myb expression.

se and with the size distribution of the colonies. The only relationship noted was the suggestion of a positive correlation between the level of myb expression and the proportion of colonies consisting of >10 cells in cells cultured in the absence of rhGM-CSF ($r = 0.75$, $P = 0.052$).

DISCUSSION

The studies described here demonstrate that the behaviour of clonogenic leukaemia cells *in vitro* is not correlated with any of the patient characteristics which were evaluated nor with any characteristic of the leukaemia cell population as a whole. These characteristics included the morphological appearance of the leukaemia cells, their cell cycle characteristics and the levels of expression of myc, myb, fms and IL1β. Surprisingly, leukaemia cell populations, which were proliferating rapidly *in vivo*, were not more highly proliferative *in vitro* than leukaemia cell populations which were proliferating slowly *in vivo*. This was the case whether the *in vivo* proliferative behaviour of the leukaemia cells was estimated by the level of the white blood cell count or by the cell cycle characteristics of the cells.

The most obvious explanation for the lack of relationship between the proliferative characteristics of the leukaemia cell population as a whole and that of the clonogenic cells is that two different cell populations are being compared. However, the lack of correlation, described here, between the ^{3}HTdR Si of the clonogenic cells and their cloning efficiency or the size of

the colonies produced *in vitro* suggests that another possible explanation may be responsible. One would expect that those leukaemia specimens whose clonogenic cells are proliferating at the most rapid rate (highest Si) would be those which produce the largest-sized colonies. As noted, this was not the case. An explanation for the apparent differences between the leukaemia cell population as a whole and those with clonogenic capacity *in vitro* is that the former assessments were made *in vivo* while the latter are made *in vitro*, and that once placed *in vitro* the properties of the leukaemia cells change.

As part of our study, we compared the clonogenic cells found in the PB and BM, with respect to primary cloning efficiency, distribution of the sizes of the clones produced, and drug sensitivity. No differences were detected (data not provided). Hence, leukaemia cells obtained from either site are suitable for making these measurements. In a similar vein, the data presented here suggest that the assessment of PE1 and the number of colonies consisting of ≥20 cells may be sufficient to provide an estimate of the secondary clonogenic capacity of AML cells. Similar data have been described previously by other investigators [13]. In fact, 'self-renewal capacity' may be a misnomer. It is possible that the PE2 does not represent self-renewal capacity, but rather provides a measure of the proliferative capacity of the clonogenic cells, that is, the number of divisions the clonogenic cell and its progeny can undergo. Since self-renewal capacity, as assessed in the system described here, rarely is continued beyond a second cloning, it is possible that the assay measures residual but declining clonogenic capacity in primitive progenitor cells, which are subsequently cloned, rather than self-renewal capacity.

With respect to clinical issues, the correlation between PE1 and PE2 in all likelihood explains why both the primary and secondary clonogenic characteristics of leukaemia cells have been reported to be prognostically important [13–16]. While it had been previously postulated that the clinical relevance of these leukaemia cell characteristics are important for 'biological' reasons [16], the inverse correlation between the number of clones consisting of ≥20 cells and sensitivity to araC and DNR demonstrate that the leukaemia cells with high proliferative capacity are likely to have a low sensitivity to chemotherapy, and thus be more difficult to kill.

In summary, the data described here demonstrate that the properties of the clonogenic cells in AML are unrelated to the characteristics of the leukaemia cell population as a whole. It was also found that the characteristics of the leukaemia cells with respect to primary and secondary clonogenicity are significantly correlated, an observation which may resolve the apparently competing claims as to whether primary or secondary clonogenicity are prognostically important.

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