Simple method for granulocyte-macrophage cell culture and staining in soft agar: comparison with a standard research technique

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SUMMARY A modified, small volume, two phase, disc culture system for CFU-GM (seven and 14 days of incubation) was compared with a standard single layer system. The 1 ml single layer cultures were counted unstained *in situ* before both sets of cultures were transferred to glass slides for staining. Bone marrows were cultured from forty eight subjects, including normal controls and patients with acute non-lymphoblastic leukaemia, acute lymphoblastic leukaemia, and myelodysplastic syndrome. Observer error was least with the disc cultures, whereas variation between replicate cultures was similar for both methods. A high degree of correlation was found between the two methods for both day 7 ($r = 0.90$) and day 14 ($r = 0.91$) cultures. The number of colonies and clusters was higher with the disc system, indicating better cloning efficiency. Analysis of subsets of clinical groups showed similar patterns of abnormality with both systems.

The simplicity of the method makes the use of this technology possible in most laboratories, and the superior morphological resolution may increase the clinical usefulness of such studies.

Clonal culture techniques of bone marrow are well established as research tools in haematology. They provide an adjunct to standard methods of assessment of haemopoiesis, giving information on the number and proliferative function of precursor cells, the earliest of which cannot be distinguished morphologically.^{1,2} The potential value of such methods in the clinical assessment of patients is considerable, but their introduction into non-specialist laboratories has been hindered by their complexity and the need for special facilities.

The basis of the clonal culture of granulopoietic progenitor cells is the proliferation of individual clones in semi-solid culture medium, stimulated by growth factors with "colony stimulating activity" (CSA). In the two layer system³ colony stimulating activity is provided by an underlayer of white cells (feeder layer), but these can be unreliable, time consuming, and inconvenient to prepare. The inclusion of medium conditioned by cells producing CSA in a single layer system has therefore become popular. Various sources are used, notably human placenta,^{4,5} a bladder cancer cell line,⁶ and giant cell tumour cells.⁷ Conditioned

medium from normal human white cells rarely provides rewarding results.

In standard research methods^{2,8,9} a single layer of medium covers the base of a 35 mm dish. Colonies and clusters are enumerated *in situ* using phase contrast of an inverted or dissecting microscope. We developed a method based on that of Kubota *et al*,¹⁰ which we believe to have several advantages over the standard method.

Material and methods

For each bone marrow sample cultures were established by the standard method and by our method. Colonies and clusters were enumerated before and after May-Grünwald Giemsa staining. The precision of the assays was compared in terms of interoperator variability and in the correlation of counts obtained by a single operator in duplicate or replicate cultures. Samples from a selection of patients with blood disorders were cultured to compare findings obtained with the two methods and with previous reports of growth patterns.

Bone marrow samples were collected from 47

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Fig 1 Clonal morphology. (a) Day 7 normal MGG colonies $\times 10$, "stained" standard method; (b) day 7 normal MGG colony $\times 10$, disc method; (c) day 14 normal MGG colony $\times 63$, disc method; (d) day 14 ANLL combined esterase stained cluster $\times 40$, disc method; (e) day 14 ANLL APAAP, transferrin receptor positive macrophages and GM colony $\times 10$, disc method.

unselected subjects including six from normal controls, 15 from patients with acute non-lymphoblastic leukaemia (ANLL), nine with myelodysplastic syndrome (MDS), three with acute lymphoblastic leukaemia (ALL), and four with chronic renal failure (CRF). The remaining clinical samples were from two patients with iron deficiency anaemia, one with idiopathic thrombocytopenia, one with lymphoma, three with reactive marrows, and three diluted bone marrow samples. The normal samples were from

subjects in whom no clinically important abnormality was found.

A quarter to one half millilitre of aspirated bone marrow was added to a transport medium of McCoy's 5A medium (modified), containing 50 U/ml preservative-free heparin, 50 U/ml penicillin, 50 cmg/ml streptomycin and 25 mM HEPES. Samples were processed within two hours of collection. The light density bone marrow cells (LDBMC) were harvested from the interface of Ficoll-Isopaque (1.077 g/cm^3

Table 1 Cloning efficiency for all methods

	Colonies			Clusters		
	Standard	"Stained" standard	Disc	Standard	"Stained" standard	Disc
Day 7:						
Mean	31	71	78	145	217	248
Median	15	68	70	117	169	237
Minimum	0	0	0	3	3	0
Maximum	154	273	380	745	1764	2435
n =	39	39	39	39	39	39
Day 14:						
Mean	46	59	73	49	70	95
Median	37	52	54	30	46	69
Minimum	0	0	0	1	1	1
Maximum	186	226	422	531	853	692
n =	40	40	40	40	40	40

Pharmacia), washed twice, and resuspended in McCoy's medium containing 15% fetal calf serum (Gibco).

For both methods, light density bone marrow cells at a final concentration of $5 \times 10^4/\text{ml}$ were cultured in McCoy's 5A medium (modified) enriched with L-asparagine (2 mg/100 ml), L-glutamine (2 mM), and containing 15% (v/v) fetal calf serum, 10% (v/v) GCT-conditioned medium (Gibco), 0.2% v/v haemolysate, 1% (v/v) penicillin/streptomycin and 0.5% v/v agar (Bacto, Difco). For exact comparability, one batch of

semi-solid medium was prepared for the two types of culture. In the standard method 1 ml was added to each 35 mm vented Petri dish (Nunc, Gibco) to completely cover the base.

In our modified single layer assay or "disc" system 200 μl aliquots for day 7 cultures and 400 μl aliquots for day 14 cultures were delivered into the centre of each dish. Small discs of 1 and 2 cm in diameter were formed and allowed to set. An identical culture medium without cells or agar (liquid phase) was then added to a total volume of 2 ml. Replicate cultures

Fig 2 Linear regression on square root transformed results for day 14, disc and "stained" standard methods (SSM) compared with standard method (SM). $\circ$ = disc, $\bullet$ = SSM

(a) Day 14 colonies ($\circ$) $r = 0.91$ $n = 47$ ($\bullet$) $r = 0.98$ $n = 40$.

(b) Day 14 clusters ($\circ$) $r = 0.95$ $n = 47$ ($\bullet$) $r = 0.96$ $n = 40$.

Table 2 Differences between observer means*

	Day 7			Day 14		
	Colonies	Clusters	n	Colonies	Clusters	n
Standard method	NS	p < 0.025	24	p < 0.01	p < 0.001	21
"Stained" standard	p < 0.001	p < 0.05	36	p < 0.001	p < 0.01	35
Disc method	p < 0.05	p < 0.05	51	NS	NS	48

*Comparison by two way analysis of variance.

Comparisons are of the means of the duplicate samples (after square root transformation) for each observer.

from both assays were incubated at 37°C for seven and 14 days in fully humidified clear plastic boxes (Gallenkamp), gassed for 10 minutes with a stream of 5% carbon dioxide and 5% oxygen in nitrogen (BOC) and sealed with pressure sensitive adhesive tape. At the end of the incubation period the standard cultures were counted immediately in situ, using an inverted microscope at $\times 40$ magnification, before being carefully floated on to a 76 $\times$ 50 mm glass slide, before staining. To prepare the disc cultures for staining, the liquid phase of each was removed and the agar disc slid on to a 26 $\times$ 76 mm glass slide. In both methods the agar medium was dried on to the slide by blotting under gentle pressure using Whatman No 9 filter paper and stained by May-Grünwald-Giemsa.¹¹ Stained cultures from both methods were overlaid with a 1 mm² grid (Graticules Ltd, Tonbridge, Kent), and scored by three observers using light microscopy at $\times 100$ magnification. Groups of more than 40 cells were classified as "colonies" and groups of 5–40 cells as "clusters".

Selected slides were also stained by combined esterase¹² and immunocytochemical (APAAP) methods.¹³

Results

Staining of cultures allowed granulocyte and macrophage clones to be distinguished, together with their stage of maturation, and made the enumeration of colonies and clusters much simpler (fig 1). Combined esterase cytochemistry and APAAP techniques could be applied to cultures (fig 1), yielding further

cytological information. Combined esterase staining of normal cultures confirms that most clones at day 7 are granulopoietic, comprising motile, chloracetate esterase positive late metamyelocytes, and earlier precursors. A few monocyte/macrophage clusters are also seen. In day 14 cultures monocyte/macrophages are more predominant and there are variable numbers of mixed lineage (myelomonocytic) clones.

Cloning efficiency (colony or cluster counts per 50 000 cells cultured) seemed to be higher in stained cultures and the highest counts were obtained with our disc system (table 1, fig 2). These differences were also seen in a plot of the difference between the two stained methods against the standard method (data not shown). A high degree of correlation between the counts for all methods was found at day 14 (fig 2). Similar results were obtained at day 7, when the standard method was compared with the disc system, for colonies ($r = 0.90$, $n = 45$) and clusters ($r = 0.79$, $n = 45$). The corresponding values for the "stained" standard method were: colonies ($r = 0.91$, $n = 40$) and clusters ($r = 0.94$, $n = 40$).

Significant differences occurred among the counts obtained by three observers counting the same material (table 2), although these differences were small in relation to the range of results (the three observer means for all methods differed by a maximum of 25 colonies, on a range of 0–300, and by 20 clusters on a range of 0–400).

Variation among observers was least with the disc system and reached only borderline significance. One

Table 3 Intra-assay variation using pooled standard deviations*

Method	Day	n†	Colonies			Clusters		
			O1	O2	O3	O1	O2	O3
Standard	7	3	0.47	0.46	0.45	0.57	0.67	0.62
	14	3	0.47	0.43	0.60	0.67	0.52	0.56
"Stained" standard	7	3	0.80	0.69	0.79	1.05	0.94	1.50
	14	3	0.52	0.61	1.03	0.70	0.56	0.94
Disc	7	2	0.51	0.45	0.46	0.86	0.66	0.53
	14	2	0.72	0.56	0.71	1.12	0.49	0.62

O = Observer.

* = SD of square rooted values.

† = Maximum number of replicate samples.

Fig 3 Comparison of CFU-GM growth by clinical type using disc and standard methods (SM) in day 7 cultures. $\circ$ = disc, $\bullet$ = SM.

way analysis of variance for each observer separately (table 3) showed that the intra-assay variation was least for the standard method and most for the "stained" standard method. Correction for differences in the range of absolute counts, by calculation of the coefficients of variation, showed that the precision of the standard and disc methods was 13% and 11%, respectively.

Colony and cluster counts obtained by the standard method were compared with those obtained by the disc method in a variety of haematological diseases (fig 3). Substantially the same patterns of growth were obtained by both methods for days 7 and 14 cultures.

Discussion

The clinical value of CFU-GM assays is well recognised.^{14,15} Methods originally developed for research have been applied to clinical problems—for example, in ANLL¹ and in MDS.¹⁶ A district general haematology laboratory considering the introduction of a CFU-GM assay, however, would need special facilities and specialised expertise for such methods.

None of the methods so far described^{2,8,9} is completely suitable, nor have we found any reports of inter-method comparisons. The disc method reported here produces results comparable with those of a standard research technique⁸ and has significant advantages in convenience and robustness.

Our method can be performed by registered MLSO's, using equipment normally available in a haematology laboratory.

Analysis of results for a range of patient samples showed that the medians, square root regression data, and plots of the differences between methods gave good correlation of the disc system with the standard method (range of correlation coefficients 0.79–0.98). The number of colonies was higher with both stained methods than with the standard method; this may have been due to colonies in stained cultures being in one plane. In the three dimensional standard method colonies might not have been observed due to focusing problems. The higher counts are unlikely to have been related to the number of dead colonies as intact colonies only were counted. It is reasonable to conclude that the higher cloning efficiencies are an indica-

tion of increased sensitivity. The superiority of our method is also indicated by its minimal intra-assay, interassay, and interoperator variation.

Grouping samples by clinical type for the standard and disc methods shows that the latter can also distinguish between normal patterns and various disease types (fig 3). Although the numbers of our subgroups were small, the findings in ANLL were similar to those reported elsewhere,¹ and presentation/relapse ALL samples usually produce suboptimal growth of clones. Our MDS and CRF subgroups seemed to show greater variation than normal and the relative excess of clusters frequently found in MDS¹⁶ was seen in our data. The same applies to the ANLL subgroup.

Although the standard research method is well accepted,^{8,17} our comparisons show that enumeration of clones is enhanced by staining of cultures. The reduction in volume of the semi-solid agar culture medium in our system means that the cultures can be mounted in toto on a standard microscope slide and stained by Romanowsky and other stains. Colonies and clusters are thus easily counted. Other workers have stained cultures,^{18,19} although none used Romanowsky stains. For example, Kubota *et al* used single esterase staining to investigate colonial architecture.¹⁰ In our Romanowsky stained cultures discrimination could easily be made between neutrophil, macrophage, and eosinophil clones and their stage of maturation.

In addition to Romanowsky morphology, our system also permits the use of cytochemical and immunocytochemical techniques for the more detailed characterisation of clones, which must be relevant to the full clinical assessment of CFU-GM assays. Although other investigators have reported the application of cytochemical^{20,21} and immunocytochemical techniques,^{22,23} none has used culture discs mounted on to standard size microscope slides. Cytochemistry provides a clearer delineation between granulocyte and macrophage clones (fig 1d). Immunocytochemistry (fig 1e) allows the expression of antigen sites on individual cells to be investigated using a range of monoclonal antibodies. Work in this area is currently in progress.

The disc system described here compares well with a standard research method, using a variety of clinical material. The simplicity of the method and the increased morphological resolution makes the use of this technology possible in most hospital laboratories. These features may also increase the clinical relevance—for example, in assessing the viability of cryopreserved bone marrow. The technique may also be useful in research—for example, in assessing potential in vitro morphological and marker changes in MDS clones grown in the presence of recombinant growth factors.

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References

- 1 Moore MAS, Spitzer G, Williams N, Metcalf D, Buckley J. Agar culture studies in 127 cases of untreated acute leukaemia: the prognostic value of reclassification of leukaemia according to in-vitro growth characteristics. *Blood* 1974;44:1-18.
- 2 Kurland JI. Granulocyte-monocyte progenitor cells. In: Golde DW, ed. *Methods in Haematology. Volume II. Hematopoiesis*. New York: Churchill Livingstone, 1984:87-122.
- 3 Pike BL, Robinson WA. Human bone-marrow colony growth in agar-gel. *J Cell Physiol* 1970;76:77-84.
- 4 Burgess AW, Wilson EMA, Metcalf D. Stimulation by human placental conditioned medium of haemopoietic colony formation by human marrow cells. *Blood* 1977;49:573-83.
- 5 Schlunk T, Schleyer M. The influence of culture conditions on the production of colony-stimulating activity by human placenta. *Exp Hematol* 1980;8:179-84.
- 6 Myer CD, Katz FE, Joshi G, Millar JL. A cell line secreting stimulating factors for CFU-GEMM culture. *Blood* 1984; 64:152-5.
- 7 Dipsio JJ, Brennan JK, Lichtman MA, Abboud CN, Kirkpatrick FH. The fractionation, characterisation and subcellular localisation of colony-stimulating activities released by the human monocyte-like cell line GCT. *Blood* 1980;56:717-27.
- 8 Testa NG. Clonal assays for haemopoietic and lymphoid cells in vitro. In: Potten CS, Hendry JH, eds. *Cell clones*. Edinburgh: Churchill Livingstone, 1985:27-43.
- 9 Bailey-Wood R, Dallimore CM, Smith SA, Whittaker JA. Use of logistic regression analysis to improve prediction of prognosis in acute myeloid leukaemia. *Leuk Res* 1984;8:667-79.
- 10 Kubota K, Mizoguchi H, Muira Y, Suda T, Takaku F. A new technique for the cytochemical examination of human hemopoietic cells grown in agar gel. *Exp Hematol* 1980;8: 339-44.
- 11 Shibata A, Bennett JM, Castolei GL, *et al*. ICSH reference method for staining of blood and bone marrow films by azure B and eosine Y (Romanowsky stain). *Br J Haematol* 1984;57: 707-10.
- 12 Shibata A, Bennett JM, Castolei GL, *et al*. ICSH Recommended methods for cytological procedures in haematology. *Clin Lab Haematol* 1985;7:55-74.
- 13 Burgess R, Hyde K, Liu Yin JA, Delamore IW. A method to evaluate the phenotype of cells in situ in CFU-GM semi solid agar cultures and its role in the evaluation of cell viability and growth. *Br J Haematol* 1989;71(Suppl 1):43.
- 14 Milner GR, Testa NG, Geary CG, *et al*. Bone marrow culture studies in refractory cytopenia and "smouldering leukaemia". *Br J Haematol* 1977;35:251-61.
- 15 Metcalf D. Detection and analysis of human granulocyte-monocyte precursors using semi-solid cultures. *Clin Haematol* 1979;8:263-85.
- 16 May SJ, Smith SA, Jacobs A, Williams A, Baily-Wood R. The myelodysplastic syndrome: analysis of laboratory characteristics in relation to the FAB classification. *Br J Haematol* 1985;59:311-19.
- 17 Borbenyi Z, Cinkotai KI, Harrison C, Testa NG. The growth of myelodysplastic bone marrow in long term cultures. *Br J Cancer* 1987;55:291-3.
- 18 Lidbeck J, Manson JC. Staining of Granulopoietic agar colonies

- with a modified Papanicolaou technique. *Exp Hematol* 1979;7:166-9.
- 19 Phillips PG, Chikkappa G, Brinson PS. A triple stain technique to evaluate monocyte, neutrophil, and eosinophil proliferation in soft agar cultures. *Exp Hematol* 1983;11:10-7.
- 20 Jacobs P, Dubovsky D, Smith S, Randall G, Bracher M. Bone marrow culture in vitro. A technique for analysis and permanent recording of cellular composition. *Exp Hematol* 1979;7:177-82.
- 21 Konwalinka G, Glaser P, Odavio R, Bogusch E, Schmalzl F, Braunsteiner H. A new approach to the morphological and cytochemical evaluation of human bone marrow CFUc in agar cultures. *Exp Hematol* 1980;8:434-40.
- 22 Davis FM, Dicke KA, Jagannath S, Rao PN. Detection of leukaemic cell colonies in agar plates by immunostaining for human malignancy-associated nucleolar antigen. *J Immunol Methods* 1983;58:349-57.
- 23 Villa S, Domenico D. Analysis of leukocyte antigens detected by immunoenzymatic staining on human marrow colonies. *Exp Hematol* 1988;16:340-3.

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