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HL-R5 AND HL-D4: TWO DIFFERENTIATION RESISTANT HL-60 VARIANTS

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Abstract—A wide range of different agents are capable of inducing the onset of HL-60 differentiation along the myeloid lineage. The diversity of these agents has made the analysis of the molecular mechanisms involved in the regulation of the onset and progress of terminal differentiation in these cells difficult. We have adapted the standard soft-agar cloning procedure to enable the single-step selection of clonal populations of spontaneously arising differentiation resistant HL-60 variants. This simple procedure which obviates the need for mutagenesis, long-term exposure to inducing agents or complex manipulations, optimises the chance of obtaining variants with a single lesion blocking the onset of differentiation. The analysis of two variants, HL-R5 and HL-D4, selected by this procedure for resistance to retinoic acid and DMSO, respectively, suggests the existence of different pathways used by the two agents which converge before the onset of terminal differentiation.

Key words: HL-60, differentiation, myeloid, DMSO, retinoic acid, variants.

INTRODUCTION

LITTLE IS known about the cellular and molecular events regulating the onset and progress of terminal differentiation in myeloid cells. The "promyelocytic" cell line HL-60 [1, 2] provides a useful *in-vitro* model for the study of myeloid and monocytic differentiation. This cell resembles in many ways an immature myeloid cell arrested in a cycle of self-renewal, with a limited rate of spontaneous differentiation in culture (approximately 10%). Agents such as DMSO [3] and retinoic acid [4] greatly enhance this background rate of differentiation resulting in the appearance of a vastly increased number of cells (more than 80%) that display a predominantly myeloid/granulocytic phenotype [3, 4]. By contrast, other agents including phorbol esters [5] and 1,25$(OH)_2$ vitamin D_3 [6] dramatically stimulate the appearance of monocyte/macrophage-like characteristics in these cells. In addition to the above agents it has been demonstrated that the careful manipulation of the pH in culture causes the appearance of eosinophile-like cells in the population [7]. Early passage HL-60 cells can also be induced to differentiate by naturally occurring factors (DIFs) in T-lymphocyte conditioned medium [8], purified colony stimulating factors (G-CSF & GM-CSF) [9, 10] gamma interferon [11, 12] and tumour necrosis factor [13] (see Ref. [14] for a recent review). The existence of such a vast array of diverse compounds all of which induce the onset of terminal differentiation in HL-60 cells has made the analysis of the processes involved very difficult. The isolation of suitable differentiation resistant subclones, each with a discrete lesion in the differentiation pathways used by the above agents, would provide a powerful tool for the analysis of the control of HL-60 differentiation.

Here we report the adaptation of the standard soft-agar cloning technique to enable the selection of differentiation resistant variants without the need for complex manipulations or mutagenesis. The procedure is based on the fact that the induction of differentiation in HL-60 cells results in the cessation of growth, whilst the spontaneously appearing differentiation resistant cells continue to proliferate and form discrete colonies in soft-agar. This technique has allowed the selection of a number of variants resistant to the induction of terminal differentiation. Here we report the characterisation of two interesting variants, HL-D4 and HL-R5.

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Abbreviations: *RA*, retinoic acid; *DMSO*, dimethylsulphoxide.

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MATERIALS AND METHODS

Cell culture

HL-60 cells in passage 28 were obtained via Dr Mingchi Wu, Biochemistry Department, North Texas State University, U.S.A. Cells were maintained below 1×10^6/ml in RPMI medium supplemented with 10% foetal bovine serum (FBS), glutamine 2 mM, pyruvate 1 mM, penicillin 200 U/ml and streptomycin 200 μg/ml. Cultures were incubated at 37°C in tissue culture flasks, initially equilibrated in an atmosphere of 95% air/5% CO_2. Routinely cells were used in passages 30–50.

For the induction of differentiation, cells in logarithmic growth (doubling time approximately 35 h) were adjusted to 4×10^5/ml in 10% FBS and an equal volume of medium containing DMSO or retinoic acid was added to the desired final concentration. Differentiation was then monitored by the daily assessment of cell number, nitroblue tetrazolium (NBT) reducing activity and morphological appearance.

Cell counts

Each point represents the mean and standard error of a single count (over 100 cells) from three independent cultures initiated under identical conditions. All counts represent viable cell number as assessed by trypan blue exclusion using a haemocytometer.

NBT reduction test

This test was performed essentially as described by Ferrero *et al.* [15]. Briefly 1×10^5 cells were washed in Ca/Mg free PBS, resuspended in 100 μl of 1 mg/ml NBT in Ca/Mg free PBS containing 1 μg/ml 12-*O*-tetradecanoylphorbol 13-acetate (TPA). After 30-min incubation at 37°C samples were placed on ice and more than 100 cells were examined using a haemocytometer. Results are expressed as the percentage of cells with intracellular blue/black formazan deposits. Each point is the average of a single determination on three independent cultures initiated under identical conditions.

Assessment of morphology

At the indicated times 1×10^5 viable cells were washed in Ca/Mg free PBS and spun onto microscope slides using a Shandon cytospin at 1000 rev/min for 5 min. Slides were air dried, fixed in 100% methanol for 10 min, stained in 50% v/v May/Grünwald for 10 min and then in 10% v/v Giemsa for 30 min. Stained slides were washed in PBS for 5 min, rinsed in tap water, counter stained for 20 min in Mayers Haemalum, rinsed in tap water, air dried and finally mounted in Depex. May/Grünwald and Giemsa were diluted freshly each time in Ca/Mg free PBS.

Isolation of differentiation resistant variants

HL-60 cells (passage 30–40) in logarithmic growth were adjusted to 2×10^5/ml in 25–50% conditioned medium and 20% FBS in the presence of either 1.25% DMSO or 1 μM retinoic acid and 0.3% noble agar. This cell suspension was added to tissue culture dishes at 20 ml per dish and the agar allowed to set at room temperature for 30 min. After 24–48 h of incubation at 37°C, cultures were examined under an inverted microscope. Typically, small colonies of between two and eight cells were visible at this time. Having confirmed the viability of the culture, a further 20 ml of medium containing 20% FBS with the appropriate inducer was added to each dish; subsequently 25% of the total volume in the dish was replaced every 3–4 days. Discrete colonies (1 mm diameter) of putative differentiation resistant variants appeared three to four weeks later. These colonies were picked out with a pasteur pipette and placed in a small volume of fresh inducer containing medium. The colonies were not disrupted and allowed to seed cells from their outer margins into the medium.

These small cultures were examined regularly, maintained at a density of less than 8×10^5/ml and expanded for cryopreservation (routinely this takes about 4–5 weeks from the time of removal from soft-agar).

RESULTS

Stability of differentiation competence

In order to determine the specificity of the selection technique the effect of soft-agar cloning and long-term growth on the differentiation competence of the HL-60 parent line was investigated. The subclone HL-60-LP was selected from a soft-agar culture of the parent line in the absence of a differentiation inducing agent. These cells were maintained in culture for six months (approximately 50 passages) before comparison with the HL-60 parent. The parent clone grows with a doubling time of approximately 35 h in 10% FBS (Fig. 1a), whilst as expected

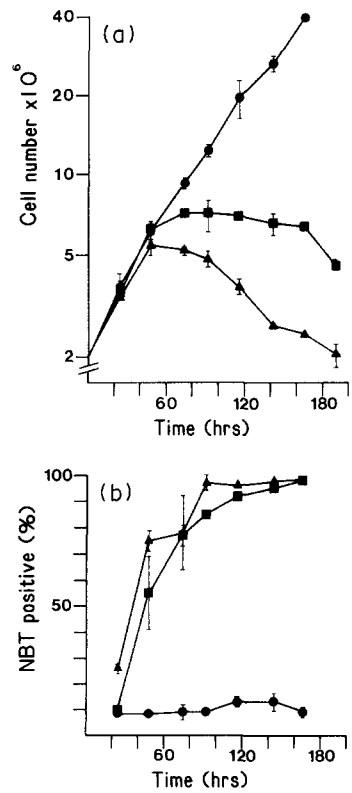

FIG. 1. Effect of DMSO and retinoic acid on the growth and NBT reducing activity of the differentiation competent HL-60 parent line. Growth kinetics (a) and NBT reducing activity (b) of cells in the presence of 1.25% DMSO (■), 1 μM retinoic acid (▲) or in the absence of inducers (●).

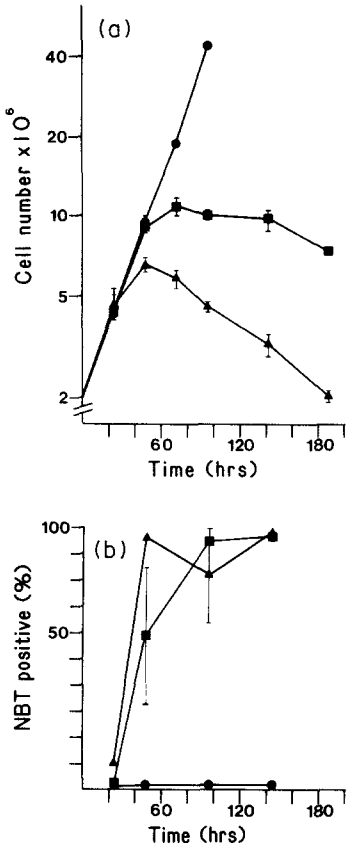

FIG. 2. Effect of DMSO and retinoic acid on the growth and NBT reducing activity of the late passage differentiation competent HL-60-LP sub-line. Growth kinetics (a) and NBT reducing activity (b) of cells in the presence of 1.25% DMSO (■), 1 μM retinoic acid (▲) or in the absence of inducers (●).

the late passage HL-60-LP has a shorter population doubling time of approximately 22 h (Fig. 2a). However, in spite of this the induced differentiation kinetics in the parent HL-60 line closely resemble those of the HL-60-LP subclone (Figs 1 and 2). In response to treatment with 1.25% DMSO they both cease proliferation after approximately 72 h (Figs 1a and 2a), and more than 80% of the cells in both populations display NBT reducing activity by 100 h (Figs 1b and 2b). When treated with 1 μM retinoic acid growth arrest becomes apparent after 48 h and the fraction of cells with NBT reducing activity exceeds 80% by 100 h. These observations demonstrate that neither the soft-agar selection, nor the long-term growth of cells in culture (fifty passages), in themselves result in the loss of differentiation competence in the HL-60 cells.

Characterisation of HL-R5

The variant HL-R5 was selected on the basis of its clonal growth in soft-agar in the continuous presence of 1 μM retinoic acid. After expansion, cryopreservation and recovery HL-R5 was cultured for 24 passages in the absence of retinoic acid, before examining its response to treatment with DMSO or retinoic acid (Fig. 3). HL-R5 characteristics which remain similar to that of the parent line include the growth arrest (Fig. 3a) and the increase in NBT (Fig. 3b) reducing activity when exposed to DMSO (compare Figs 1 and 3) and its doubling time in the control inducer free medium. By contrast, 1 μM retinoic acid fails to induce a reduction in rate of proliferation of HL-R5 cells for approximately 100 h and the NBT activity increases at a slower rate than the wild-type HL-60 cells. This resistance to the induction of differentiation by 1 μM retinoic acid is complete in 0.1 μM retinoic acid (Fig. 4). At this concentration the parent HL-60 cells growth arrest by 72 h (Fig. 4a) and by 140 h the fraction of cells with NBT reducing activity exceeds 80% (Fig. 4b). In HL-R5 cultures even after one month of exposure to 0.1 μM retinoic acid there is no sign of growth arrest (data not shown). In these cultures the fraction

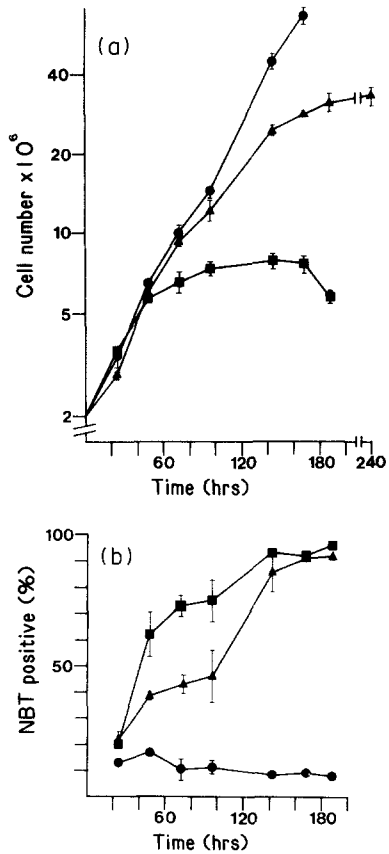

FIG. 3. Effect of DMSO and retinoic acid on the growth and NBT reducing activity of the variant HL-R5. Growth kinetics (a) and NBT reducing activity (b) of cells in the presence of 1.25% DMSO (■), 1 μM retinoic acid (▲) or in the absence of inducers (●).

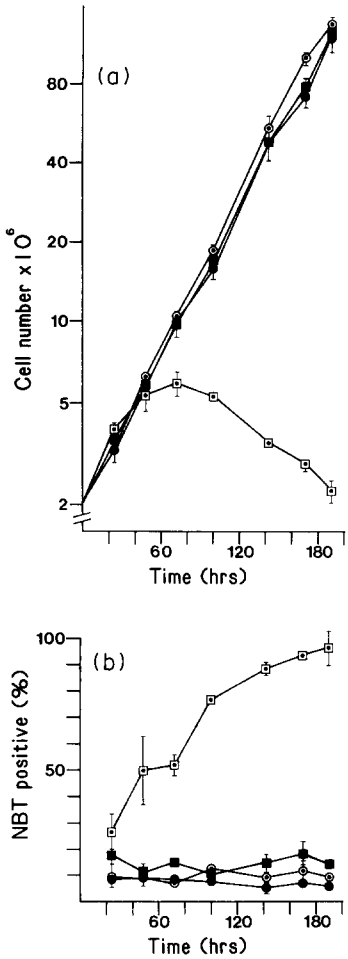

FIG. 4. Comparison of the effect of retinoic acid on the growth and NBT activity of the differentiation competent HL-60 parent line and the variant HL-R5. Growth kinetics (a) and NBT reducing activity (b) of HL-60 parent cells in the presence of 0.1 μM retinoic acid (⊡), or in the absence of the inducer (■). HL-R5 cells in the presence of 0.1 μM retinoic acid (⊙), or in the absence of the inducer (●).

of NBT reducing cells remains at the background level of 5–15%.

Characterisation of HL-D4

The HL-D4 variant was selected on the basis of its clonal growth in soft-agar in the continuous presence of 1.25% DMSO in conditions which were otherwise the same as that described for HL-R5. In the absence of inducing agents this variant grows at a similar rate (compare Fig. 5a with 1a) to that of the parent line and with a background NBT reducing activity of 4–8% (Fig. 5b). The addition of 1.25% DMSO to HL-D4 results in a small transient increase in the percentage of NBT reducing cells (rising to a peak of about 25% at about 100 h) which subsequently decline to 8–10%. In 1.25% DMSO HL-D4 cells do not become post-mitotic. These cells have been grown continuously for periods in excess of two months in 1.25% DMSO (doubling time 34 h in the untreated cultures compared to 48–53 h in the DMSO treated cultures). Interestingly, at higher DMSO concentrations there is evidence of increasing induction of differentiation in the HL-D4 cells. Whilst there is a linear increase in the fraction of NBT reducing cells in the HL-60 cultures, in HL-D4 there is little evidence of induction of differentiation at concentrations lower than 1.25% DMSO (Fig. 6). However, at 1.55% DMSO the fraction of NBT reducing cells in HL-D4 is comparable to that of HL-60 cultures in 1.15% DMSO. Therefore, the resistance of HL-D4 to the induction of NBT reducing activity by DMSO is not a consequence of an aberration in the NBT reducing metabolism in these cells, but the inability of DMSO to induce differentiation at concentrations which does result in differentiation of the HL-60 parent clone. This is further demonstrated by

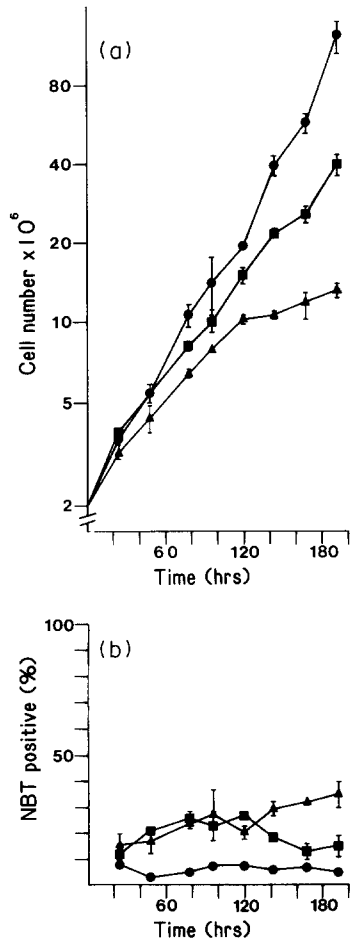

FIG. 5. Effect of DMSO and retinoic acid on the growth and NBT activity of the variant HL-D4. Growth kinetics (a) and NBT reducing activity (b) of cells in the presence of 1.25% DMSO (■), 1 μM retinoic acid (▲) or in the absence of inducers (●).

FIG. 7. Morphological appearance of differentiation competent and the resistant variants HL-R5 and HL-D4. HL-60 parent cells uninduced (a), or treated for 190 h with either 1.25% DMSO (b) or 1 μM retinoic acid (c). HL-D4 cells uninduced (d), or treated for 190 h with either 1.25% DMSO (e) or 1 μM retinoic acid (f). HL-60-LP cells uninduced (g) or treated for 190 h with either 1.25% DMSO (h) or 1 μM retinoic acid (i). HL-R5 cells uninduced (j), or treated for 190 h with either 1.25% DMSO (k) or 1 μM retinoic acid (l). Original magnification × 160.

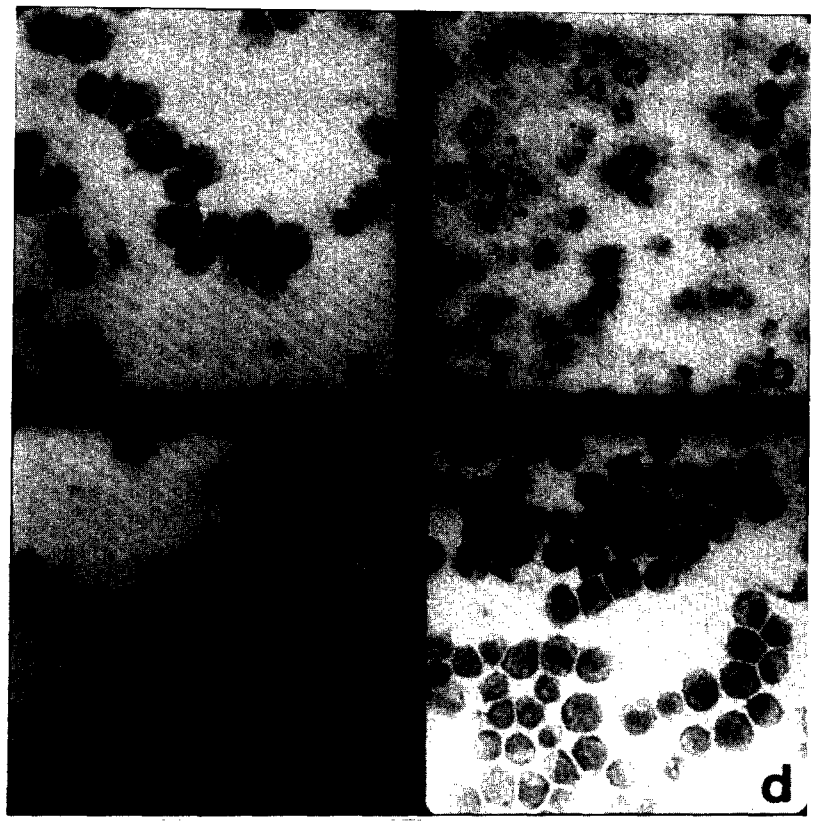

FIG. 8. Morphological comparison of HL-60 parent cells and HL-R5 treated with 0.1 μM retinoic acid. HL-60 (a) and HL-R5 cells (c) in the absence of the inducer, or treated with 0.1 μM retinoic acid for 190 h; HL-60 (b), HL-R5 (d).

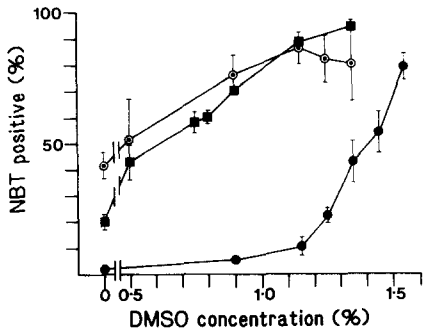

FIG. 6. The dose response of HL-D4 to DMSO alone, or in combination with retinoic acid as assayed by NBT reducing activity. HL-D4 cells were treated for three days with the indicated concentrations of DMSO either alone (●) or together with 1 μM retinoic acid (⊙). For comparison HL-60 cells were cultured with the indicated concentrations of DMSO alone (■).

the effect of increasing concentrations of DMSO when HL-D4 is cultured in the presence of a constant 1 μM retinoic acid concentration. Under these conditions the fraction of NBT positive cells in HL-D4 cultures is comparable to that induced in HL-60 by similar DMSO concentrations in the absence of retinoic acid (Fig. 6). Therefore, the combination treatment of HL-D4 with 1.25% DMSO and 1 μM retinoic acid, results in the induction of differentiation with near normal kinetics (growth and morphology data not shown). However, retinoic acid alone does not induce differentiation in HL-D4. In response to 1 μM retinoic acid even after 200 h of culture only 35% of the cells (in some experiments about 50%) show NBT reducing activity (Fig. 5b). The continuation of these cultures for a further 160 h failed to significantly increase the fraction of NBT positive cells. We have also continuously grown HL-D4 for periods in excess of one month in the presence of 0.1 μM retinoic acid without any sign of termination of growth or induction of differentiation. These properties are in marked contrast to the rapid induction of differentiation by retinoic acid in the wild-type HL-60 parent clone (see Fig. 2).

Effect of DMSO and retinoic acid on cell morphology

In the absence of inducers of differentiation both HL-60 parent and the HL-60-LP clone (Figs 7a and g respectively) show the characteristic histological features, including large round regular nuclei and grainy cytoplasms. After 190 h in the presence of 1.25% DMSO (Figs 7b and h), or 1 μM retinoic acid (Figs 7c and i) both appear to have irregular shaped cells, smaller lobular nuclei and less grainy cytoplasm. Comparison of HL-D4 with either of the differentiation competent clones in the absence of the inducers shows HL-D4 to have a slightly smaller nucleus and less grainy cytoplasm (compare Fig. 7d with 7a and 7g). After treatment with 1.25% DMSO HL-D4 still retains the regular nuclear shape (Fig. 7e) and differs markedly from the treated HL-60 parent (Fig. 7b). Similarly, after treatment with 1 μM retinoic acid (Fig. 7f) these cells resemble much more closely the untreated cultures (Figs 7a, d, g and j) than they do the treated differentiation competent clones (Figs 7c and i).

In the absence of DMSO or retinoic acid HL-R5 cells (Fig. 7j) look indistinguishable from the HL-60 parent (Fig. 7a), and treatment with 1.25% DMSO clearly results in the appearance of well differentiated cells (Fig. 7k). However, when HL-R5 cells are cultured in the presence of 1 μM retinoic acid (Fig. 7l) there is a much reduced evidence of differentiation; a large percentage of the cells retain large and relatively regular nuclei.

When the HL-60 parent and the HL-R5 variant are treated with 0.1 μM retinoic acid there is a much more pronounced demonstration of the resistance of HL-R5 to the induction of differentiation by retinoic acid (Fig. 8). The HL-60 parent clone appears well differentiated after 190 h of exposure to 0.1 μM retinoic acid (Fig. 8b). By contrast HL-R5 cells similarly treated (Fig. 8d) appear indistinguishable from those untreated (Fig. 8c) or the control HL-60 cells (Fig. 8a). Therefore, the morphological appearance of these cells are concurrent with the growth and NBT reducing activity data presented above.

DISCUSSION

The availability of a suitable panel of resistant variants in which a single genetic lesion blocks the onset of differentiation in otherwise proficient HL-60 cells would be a powerful tool for the analysis of the molecular mechanisms involved in the differentiation of these cells. A number of laboratories have reported the isolation of spontaneously arising HL-60 variants. These appear to represent, earlier, less mature myeloid cells [16, 17], and the origin of at least one such line is now in doubt [17, 18]. In addition several differentiation resistant HL-60 variants have been isolated following either mutagenesis [19–22], or continuous culture for extended periods in the presence of increasing concentrations of an inducing agent [23–25]. Both procedures increase the risk of multiple genetic lesions, due to high mutation rates and amplifications of large DNA segments which could span several genes [22]. In addition, the variants should ideally be selected from early passage, fully differentiation competent cultures in order to reduce the risk of accumulation of multiple

lesions as is likely to be the case in very late passage cultures [36, 37]. The cloning procedure described here enables the single-step isolation of a large number of clonal populations of differentiation resistant variants which arise spontaneously in cultures exposed to a single optimum dose of an inducing agent. This simple procedure, which obviates the need for mutagenesis or continuous culture in increasing concentrations of the inducing agents, optimises the chance of obtaining variants with a single lesion and no DNA amplifications.

Using the conditions described here clonal populations of cells resistant to the induction of differentiation by either DMSO or retinoic acid appear with a frequency of approximately 10^{-5}. This high frequency is probably due to the presence of the documented aneuploidy in the HL-60 cells [2], possible functional haploidism, and the involvement of multiple steps in the induction of differentiation by each of the two agents tested. The karyotype of our parent HL-60 clone, which is very similar to the karyotype published by Nowell *et al.* [38], confirms the aneuploidy of our parent HL-60 cells. They have monosomy 5, 9, 16 and 17 with a single copy of the X-chromosome. In addition there may be small deletions in the long arm of chromosome 5 and the short arm of chromosome 9. They have trisomy 10 and 18, plus three "marker" chromosomes, as well as an average of approximately 20 "double-minute" chromosomes. There is no detectable karyotypic difference between HL-60 and HL-D4 (HL-R5 not karyotyped) (unpublished data).

Because DMSO and retinoic acid are chemically different agents, they would be expected to act, initially, on different pathways. However, since exposure to either agent does result in the ultimate appearance of the same properties characteristic of mature myeloid cells, the two initial pathways probably converge and share common steps leading to the induction of differentiation. Analysis of HL-R5, demonstrating resistance to retinoic acid and sensitivity to DMSO induction of differentiation, suggests the lesion in these cells to be early in the induction pathway. That is up-stream of the point of convergence of the two pathways which may be used by these agents. By contrast HL-D4 (selected for DMSO resistance alone) shows not only resistance to DMSO but also retinoic acid induced differentiation. The lesion in HL-D4 would therefore appear to be beyond the point of convergence of the two pathways. This is of course based on the assumption that the differentiation resistance of HL-D4 is the product of a single lesion. Interestingly, 12-*O*-tetradecanoylphorbol-13-acetate (TPA) which does induce monocytic differentiation in HL-60 and HL-R5, does not induce differentiation and appears toxic to HL-D4 (data not shown). The nature of the differentiation lesion in these cells, their topological relationship to each other and their dominant or recessive character can be tested by somatic cell hybridisation mediated complementation analysis. These studies are currently in progress.

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