Terminal Differentiation of Murine Erythroleukemia Cells: Physical Stabilization of End-stage Cells

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ABSTRACT   An important limitation in the use of the murine erythroleukemia (MEL) cell system as an in vitro system for the study of terminal erythroid differentiation has been the inability to produce significant numbers of cells which represent the end-point of the pathway in vitro. We show here that a major reason for the failure to observe end-stage cells in vitro is that such cells are physically unstable under the standard culture conditions used for MEL cell differentiation. Modification of these culture conditions by the addition of either bovine serum albumin or Ficoll leads to physical stabilization of end-stage cells. Under such culture conditions, uniform cultures of terminally differentiated MEL cells with morphological characteristics similar to those of normal mouse orthochromatophilic erythroblasts and reticulocytes are observed. Examination of physical and biochemical parameters of these cell populations give values which are similar to values characteristic of mouse reticulocytes. A physically stabilized MEL cell shows a narrow cell volume distribution with an average value of $\sim 100~\mu m^3$, similar to the cell volume distribution observed for mouse reticulocytes, while a typical MEL cell culture treated with DMSO but without a stabilizing agent exhibits a broader, more heterogeneous cell volume distribution with an average value of $\sim 500~\mu m^3$. Globin mRNA levels and levels of globin synthesis reach values almost equal to those in mouse reticulocytes in cultures of physically stabilized MEL cells while differentiating cultures not treated with a stabilizing agent reach substantially lower values for these parameters. We suggest that the ability to produce populations of MEL cells which undergo complete terminal erythroid differentiation in vitro will allow the analysis of the molecular mechanisms which control the terminal stages of the erythroid differentiation process.

The in vitro differentiation exhibited by murine erythroleukemia (MEL) cells has served as a valuable model system for analyzing many important aspects of the erythroid differentiation process (5, 14). A major drawback to this system has been that in vitro differentiation does not appear to reach the final stages of the normal erythroid differentiation pathway. A number of possible explanations for the apparently incomplete in vitro differentiation of MEL cells can be proposed. It is possible that culture conditions used for the propagation of MEL cells prevent completion of the differentiation program. Alternatively, the genetic program expressed by MEL cells may not reflect the complete program of normal erythroid differentiation.

It has been reported by Ikawa et al. (9), Friedman and Schildkraut (3), and Tsiftsoglou et al. (16) that small numbers of reticulocytes can be observed in certain MEL cell cultures.

These observations suggest that the former explanation may be correct and that terminal differentiation may be promoted or stabilized by an alteration in culture conditions.

In the course of biochemical experiments on MEL cells we observed that fully differentiated MEL cells can be easily lysed by washing with physiological saline or culture medium without serum. This observation suggested to us that terminally differentiated MEL cells may be particularly fragile in the absence of serum. We reasoned that if culture conditions could be devised which protect the terminally differentiated cell from lysis, then relatively pure populations of end stage erythroid cells could be produced in the MEL cell system. The ability to manipulate the MEL cell system so that end-stage cells can be produced would be valuable for many types of studies.

We have found that such populations of MEL cells can actually be obtained by addition of an appropriate amount of

bovine serum albumin (BSA) or Ficoll (obtained from Pharmacia Fine Chemicals, Uppsala, Sweden) to the culture medium of differentiating MEL cells. In this report we have analyzed in detail the factors which are significant in obtaining terminally differentiated erythroid cells in the MEL cell system and present evidence which supports the view that stabilization of fragile cells is a key factor in the production of such cell populations.

MATERIALS AND METHODS

Cell Growth

Cell line 745 was originally obtained from Dr. C. Friend of Mount Sinai School of Medicine, New York. Freshly cloned cells, clone 745-PC-4-7, a subclone of this line, with very high probability of commitment as detected by plasma clot assay (4), was used in these experiments. Cultures were maintained in medium lacking nucleosides (α^-) and supplemented with 13% vol/vol heat-inactivated fetal calf serum (FCS; Gibco Laboratories, Grand Island Biological Co., Grand Island, NY). Cells were grown in a humid CO_2 incubator (5% CO_2) at 37°C. Cells were maintained in culture for no more than 20 generations and then replaced by frozen cells of the same clone. Erythroid differentiation was induced by addition of dimethyl sulfoxide (DMSO) (MCB Manufacturing Chemicals, Inc., Cincinnati, OH) to 1.8% to newly subcultured cells at a density of 5–10 × 10^4 cells/ml. Where indicated in text, BSA (Sigma Chemical Co., St. Louis, MO; prepared as described by McLeod et al. and adjusted to pH 7.4 with HEPES-NaOH) and iron-dextran complex were also added to the culture. In these experiments cells were grown in 3% BSA for 1–2 d before induction. Cell number and cell volume distribution were determined with an automatic cell counter, equipped with channelyzer (Coulter Counter, model ZBI, Coulter Electronics, Inc., Hialeah, FL).

Labeling of Cells with [^{14}C]Thymidine

Cells were labeled for 6 h with 0.5 μCi/ml [methyl-^{14}C]thymidine (Amersham Corp., Arlington Heights, IL; 55 Ci/mmol), then concentrated, washed, resuspended in fresh α^- medium, and divided into three cultures. These cultures were treated as indicated in the legend to Fig. 1. To measure the incorporation of radioactive thymidine into DNA, cells were harvested by centrifugation and lysed in buffer containing 50 mM HEPES, pH 8.0, 50 mM NaCl, 5 mM $MgCl_2$, 0.25 M sucrose, and 0.5% Triton X-100. Nuclei were then separated by centrifugation at 3,000 rpm for 10 min, lysed with 2% SDS, sonicated, and [^{14}C]thymidine remaining in TCA-precipitable form was determined in a Beckman scintillation counter (Beckman Instruments, Inc., Electronic Instruments, Schiller Park, IL).

Preparation of RNA and Hybridization with Globin cDNA

MEL cell cytoplasmic RNA was prepared as described by Aviv et al. (1). Mouse reticulocytes and reticulocyte RNA were prepared from BALB/c mice by standard techniques (15). Poly(A)-containing RNA was prepared as described by Aviv and Leder (2). Hybridization with tritiated globin cRNA (sp act 2.8 × 10^7 cpm/μg) was carried out as described by Ross et al. (15). The reaction was terminated by treatment with S1 nuclease as described by Housman et al. (7).

Freezing of MEL Cells

1–2 × 10^7 MEL cells were concentrated by centrifugation to a final volume of 1 ml in α^- medium containing 25% fetal bovine serum and 10% DMSO. The cells were frozen at a rate of 1°C/min and stored in liquid nitrogen.

RESULTS

To directly test the hypothesis that significant cell lysis was occurring during terminal differentiation of MEL cells in vitro, the experiment shown in Table I was performed. Cells were labeled with [^{14}C]thymidine for a period of 6 h in the absence of inducer. These cells were carefully washed to remove unincorporated [^{14}C]thymidine (TdR), and the culture was divided into three portions. In all cases cell density was maintained below 2 × 10^6 by addition of the appropriately supplemented culture medium. One aliquot was incubated in standard culture

medium in the absence of inducer. Over a 7-d period the stably incorporated [^{14}C]TdR present in a standard sample of this culture decreased no more than 10%. A culture treated with DMSO under standard conditions for induction of MEL cell differentiation showed no significant decrease in [^{14}C]TdR up to day three. However, between day four and day seven of DMSO treatment, the period during which the maximum number of highly differentiated erythroblasts are observed in these cultures, a significant decrease in [^{14}C]TdR levels per aliquot is observed. By day 7, >60% of the incorporated [^{14}C]TdR is no longer present in acid-precipitable form. These results are consistent with the view that significant cell lysis has occurred in the period following 3 d of DMSO treatment. We have examined a variety of treatments designed to physically stabilize end stage erythroblasts in this system. The effects of one such treatment on the [^{14}C]TdR levels are also shown in Table I. It is clear that 90% of the [^{14}C]TdR remains TCA-precipitable during the course of 7 d of DMSO treatment when 3% BSA is added to the medium.

To further analyze the properties of terminally differentiating MEL cells treated with BSA, a series of experiments was performed in which the morphological characteristics of DMSO-treated MEL cells stabilized by BSA treatment were monitored.

A field of cells from a culture treated with DMSO for 5 d to which neither BSA nor Ficoll has been added is shown in Fig. 1A. This field is typical of preparations made from such cultures in the sense that although almost all the cells are benzidine positive and hence contain considerable hemoglobin, a wide variation in the morphological characteristics of the cells is observed. In the field shown here, larger cells with a relatively low degree of condensation of chromatin are present in significant numbers. Only one or two cells exhibit a phenotype in which hemoglobin staining is most intense and chromatin is highly condensed. Examination of many fields from cultures similar to the one shown here reveals that, although

TABLE I

Stability of Cells Prelabeled with [^{14}C]Thymidine Grown under Various Culture Conditions

Time of induction	% of acid precipitable ^{14}C		
	DMSO	DMSO and BSA	Uninduced
h			
0	100	100	100
12	97	97	99
24	98	96	99
36	95	96	98
48	95	98	97
60	93	94	99
72	90	97	95
84	78	95	98
96	67	97	96
108	37	91	97
120	35	95	98
144	29	93	96

Cells were labeled for 6 h with 0.5 μCi/ml [methyl-^{14}C]thymidine (Amersham Corp., 55 mCi/mmol), then concentrated by centrifugation, washed, and resuspended in fresh medium, and divided into three subcultures. One culture was treated with 1.8% DMSO, a second culture was treated with 1.8% DMSO in medium supplemented with 4% BSA and 2 × 10^{-3} M Fe in the form of iron-dextran complex, while a third culture was resuspended in medium alone. At each time indicated in the table, an equal aliquot of the culture was harvested by centrifugation, nuclei were isolated, lysed, sonicated, and [^{14}C]thymidine remaining in acid-precipitable form was determined, as described in Materials and Methods.

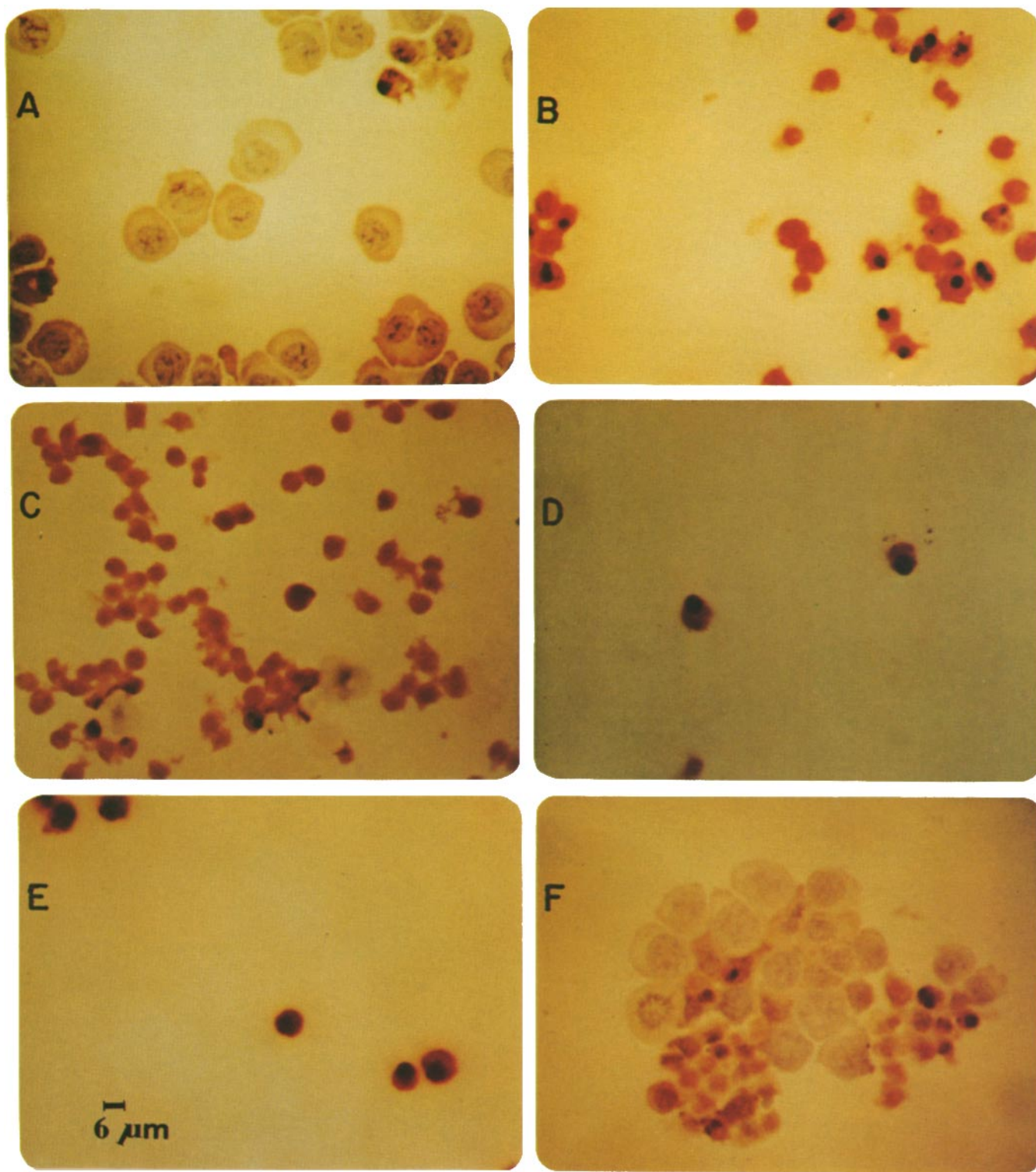FIGURE 1   Morphology of terminally differentiated MEL cells. Cells, clones PC4(F) or PC4-7(A−E), were treated for 6 d with 1.8% DMSO in α⁻ medium with 13% FCS and 5 × 10⁻³ M iron-dextran, supplemented with: A, no addition; B, 2.5% Ficoll-400; C, 5% BSA; D, 3% BSA; E, 3% BSA; and F, 5% BSA. Each photograph is of a cytocentrifuge preparation of cells stained with benzidine and counter-stained with hematoxylin as described (13).

the proportions of different morphological phenotypes vary from culture to culture, a similar heterogeneous distribution in the morphology of the cells is characteristic of such cultures. By contrast, cells derived from cultures to which Ficoll or BSA has been added exhibit a much more uniform morphology. In

Fig. 1 B, a field of cells from a culture treated with DMSO and 2.5% Ficoll for 6 d is shown. All of the cells in this field exhibit nuclei with highly condensed chromatin or are actually enucleated. All show intense benzidine staining. Treatment with DMSO and 5% BSA (Fig. 1 C) gives rise to cultures in which

most of the cells contain high levels of hemoglobin and are enucleated, whereas treatment with 3% BSA (Fig. 1 D and E) gives rise to cultures in which almost all cells have high hemoglobin levels but retain their nuclei. The nuclei exhibit the highly condensed chromatin typical of normal orthochromatophilic erythroblasts at the stage just before enucleation.

A technical problem which must be monitored with extreme care to achieve uniform cultures of terminally differentiating cells in this system is overgrowth by a population of cells which do not respond to inducer. In general, we found that the most effective means to circumvent this problem is to use a freshly cloned population of cells which exhibit a very high proportion of committed cells upon exposure to inducer. To facilitate experimental manipulations, we freeze multiple aliquots of such a population under conditions where a high proportion of cells (>70%) recover from freezing (as described in Materials and Methods) and perform experiments within a few days after such recovery. If, however, a population of cells in which even a relatively small number of cells are refractory to the effects of inducer is used, results similar to those shown in Fig. 1 F may be obtained. In this field we observe two morphological phenotypes despite the fact that the medium has been supplemented with 5% BSA. Against a background of cells with an appearance similar to that of the cells shown in Fig. 1 C (with considerable hemoglobin and enucleated) we observe a group of larger cells with uncondensed chromatin. Since the enucleated cells no longer have proliferative capacity while the less mature cells are clearly multiplying, it is apparent that in such a culture the less differentiated cells will overgrow the terminally differentiated population. For the production of uniform populations of end-stage cells to study the biochemistry of the terminal differentiation process, such a situation is clearly undesirable. As discussed above, the use of a freshly cloned, highly responsive cell population in our hands eliminates this possibility.

Analysis of the cell volume distribution of these cultures in comparison to a culture treated with DMSO alone is shown in Fig. 2. It is clear that the modal cell volume (MCV) of the BSA-treated culture is ~100 μm^3—similar to the MCV of normal mouse reticulocytes. The volume distribution is quite narrow, indicating a relatively uniform cell population. By contrast, a cell population treated with DMSO alone has an MCV of ~500 μm^3. The volume distribution of this cell population is much broader than that of the BSA-treated culture, indicative of heterogeneity in this population.

The physical stabilization of MEL cells may be due to a specific factor present in our preparation of BSA or to a more general property of BSA. To distinguish these alternatives we performed a series of experiments in which Ficoll has been substituted for BSA. This series of experiments (Fig. 1 B; and V. Volloch and D. Housman, unpublished observations) indicate that Ficoll is an effective substitute for BSA in physical stabilization of MEL cells.

Our observations on cell morphology and volume suggested to us that addition of the BSA or Ficoll to the culture medium had the effect of preserving the physical integrity of differentiated MEL cells. An issue of great significance in the use of MEL cells as an an vitro model for erythroid differentiation is the biochemical characteristics of the differentiated cell population. In most previous reports in which the levels of globin synthesis or globin mRNA have been determined in DMSO-treated MEL cells, these levels have not reached the level of reticulocytes. The levels obtained by us for these parameters in MEL cells treated with DMSO alone are shown in Fig. 3. The values we obtain are quite typical of those reported previously and are at least four to five times lower than the values obtained for these same parameters in authentic mouse reticulocytes. For example, hybridization with globin cDNA of total cytoplasmic RNA from 745-PC-4 cells treated with DMSO alone gives a half R_{ot} value of 1.5. Cytoplasmic RNA isolated from mouse reticulocytes gives a half R_{ot} value of $\sim 1.5 \times 10^{-1}$, indicative of a content of globin mRNA tenfold higher than that of the DMSO-treated MEL cells. Cytoplasmic RNA isolated from MEL cells treated with DMSO and BSA gives a half R_{ot} value of 0.95×10^{-1} when hybridized to globin cDNA. This result indicates that the globin mRNA level in these cells is almost equal to the value observed in reticulocytes. A similar relationship in the globin mRNA content of poly(A) + RNA is observed when MEL cells treated with DMSO alone are
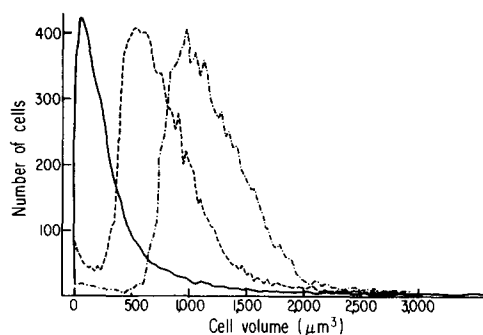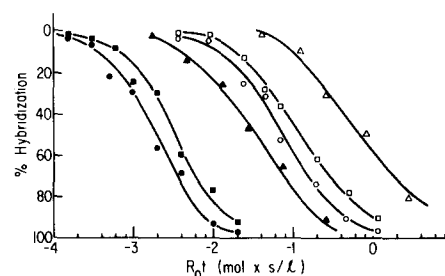

FIGURE 2 Cell volume distribution of MEL cells under different culture conditions. Cell volume was determined in three different cultures. Exponentially growing MEL cells in α^- medium. (· — ·). Cell treated with 1.8% DMSO for 4.5 days in α medium (--). Cells treated with 1.5% DMSO for 5.5 d in a medium supplemented with 4% BSA and 2 × 10⁻³ M iron-dextran (———). Cell volume was determined using an automatic cell counter (Coulter counter Model ZBI) equipped with pulse height channelyzer.

FIGURE 3 Analysis of globin mRNA levels by hybridization to globin cDNA. MEL cells were treated with 1.8% DMSO for 96 h in α medium, +13% FCS for 150 h in the same medium supplemented with 5% BSA and 2 × 10⁻³ M Fe in the form of iron-dextran complex. Cytoplasmic RNA from these cells was purified as described in Materials and Methods. A sample of this RNA was subjected to oligo(dT) cellulose affinity chromatography. The hybridization reaction with [³H]globin cDNA (1,000 cpm, 2.4 × 10⁷ cpm/µg) was carried out as described in Materials and Methods, and the percentage of the radioactive globin cDNA hybridized was determined by sensitivity to S1 nuclease as described in Materials and Methods. Mouse reticulocyte total cell RNA (○). Mouse reticulocyte poly(A)+ RNA (●). Cytoplasmic RNA from MEL cells treated with DMSO in medium supplemented with BSA (□). Cytoplasmic poly(A)+ RNA from MEL cells treated with DMSO in medium supplemented with BSA (■). Cytoplasmic RNA from MEL cells treated with DMSO in regular medium (△). Cytoplasmic poly(A)+ RNA from MEL cells treated with DMSO in regular medium (▲).

compared to mouse reticulocytes and MEL cells treated with DMSO and BSA. Half R_0t values of 3×10^{-1}, 8×10^{-3}, and 6×10^{-3} are observed when poly(A) + RNA from these cell populations are hybridized to globin cDNA. Treatment of MEL cells with DMSO in the presence of 3% BSA leads to a level of globin synthesis of ~85% of total cellular protein synthesis, a value comparable to the level of ~90% of cellular protein synthesis observed in reticulocytes. Similarly, globin mRNA levels in MEL cells treated with DMSO + BSA reach a proportion of 1.5–2% of cellular RNA. This value is comparable to the value observed for reticulocyte RNA. By contrast, globin mRNA represents only 0.2–0.3% of the total cellular RNA in cells treated with DMSO alone. These results are again consistent with the view that addition of BSA to the medium physically stabilizes terminally differentiated MEL cells.

The experiments on levels of globin mRNA were performed on cells treated with DMSO or with DMSO + BSA at times at which maximum values for these parameters are observed under each growth condition. Subsequent to 4 d of exposure to DMSO, lysis of differentiated cells and overgrowth of nonresponding cells actually reduce the proportion of globin mRNA and the proportion of globin synthesis in these cells. By contrast, cultures in which end-stage cells are stabilized with BSA continue to show increases in the proportion of globin mRNA and globin synthesis until 6 d after the addition of inducer.

DISCUSSION

The demonstration that MEL cells induced with DMSO to differentiate give rise to a physically unstable population of terminally differentiated cells has a number of significant implications. In particular, it appears essential to reevaluate studies on the biochemical properties of DMSO-treated MEL cells to determine whether particular measurements have been influenced by the lysis of end-stage cells.

For example, it has been reported that globin mRNA is unstable in MEL cells treated with DMSO for 4 d (12). Globin mRNA is reported to exhibit an apparent half-life of 17 h in such cells. It is possible, however, that the apparent instability of globin mRNA in DMSO-treated MEL cells is a reflection of the physical instability of the cells themselves. We have obtained data which support this view and in fact indicate that globin mRNA is actually more stable than other cellular mRNAs (17). This result would account for the high levels of globin synthesis and globin mRNA observed in the physically stabilized MEL cells characterized for these parameters in our study.

The control mechanisms which govern other aspects of the differentiated phenotype are now more amenable to study in the MEL cell system. For example, reticulocytes are known to exhibit a fourfold higher level of $tRNA_{his}$ than other cell types. Using MEL cells stabilized by the procedures described here,

we have been able to reproduce this increase in $tRNA_{his}$ levels characteristic of erythroid differentiation (M. Litt, V. Volloch, and D. Housman, manuscript in preparation). These results open the possibility of the use of the MEL cell system for analysis of the control mechanisms which are responsible for this shift in the tRNA spectrum.

Other studies of the terminal differentiation process should also be possible using the techniques described here. In particular, the analysis of the differentiation of the specialized erythrocyte cell membrane should be facilitated by the availability of a reliable in vitro system. Similarly, studies directed towards understanding the mechanism of chromatin condensation and enucleation in differentiating erythroblasts should be feasible in the MEL cell system. Finally, the coordination mechanisms which integrate various aspects of the differentiation process may now be more amenable to study in the MEL cell system.

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