ABSTRACT

Only two chemicals (transferrin and selenium dioxide) are required to supplement serum-free Roswell Park Memorial Institute Medium 1640 for long-term growth and for spontaneous and induced differentiation of established lines of human and mouse erythroleukemia cells. We describe here two serum-free media (a minimal synthetic medium and a high-density synthetic medium) that support the growth and differentiation of human K562(S) and mouse clones 745, 707, and 3TCl 12 erythroleukemia cell lines in long-term culture. The doubling times of the erythroleukemic cell populations are longer in minimal synthetic medium than in serum-containing medium. Cell saturation density in minimal growth medium is one-half that obtained in serum-containing medium for clone 745, whereas for K562(S) it is approximately the same. Cell saturation density in high-density medium (containing albumin) is greater than that achieved in serum-containing medium for K562(S), whereas for clone 745 cell saturation density increases for cells in midlogarithmic growth, although not to the density of cells grown in serum-containing medium. The differences in saturation density are due to a decreased doubling time as well as to better survival of the cells 3 or 4 days after plating. The cells can grow in the synthetic medium and be passaged for as many generations as desired without impairment of growth capabilities. In the minimal synthetic medium, spontaneous differentiation of erythroleukemia cells continues to occur, indicating that spontaneously differentiating cells are the result of intracellular mechanisms controlling the expression of a genetic program of some of the cells at any given time. Hemoglobin synthesis can be induced in cells growing in synthetic medium by using lower concentrations of the same inducers that are effective in serum-containing medium, indicating that these chemicals do not depend on serum factors to initiate the process of differentiation. The percentage of benzidine-positive cells and the concentration of hemoglobin per cell, however, are less in the synthetic medium than in serum-containing medium, suggesting that serum factors do play a role in modulating the extent of hemoglobin synthesis. The types of hemoglobins synthesized by cells in synthetic medium are identical to those reported in serum-containing medium.

INTRODUCTION

Human and mouse erythroleukemia cell lines are being used extensively to study the processes associated with terminal erythroid differentiation in vitro. These erythroleukemia cell lines are all characterized by the presence of a spontaneously differentiating fraction of cells when grown in serum-containing medium (2, 3, 6, 9, 21, 22, 26) and by the ability to differentiate and synthesize hemoglobins when they are grown in the presence of a variety of chemical compounds (for reviews, see Refs. 1, 15, and 19).

It is not clear whether the serum factors totally or partially mediate the spontaneous differentiation observed in a fraction of the population growing in the serum-containing medium or, rather, that the differentiative program is regulated and expressed through intracellular mechanisms. It is also not clear whether all the inducers of differentiation studied thus far act directly on the cells or instead sensitize the cells to factors present in the serum. We recently reported that human erythroleukemic K562(S) cells (18) can be grown continuously in Breitman’s synthetic medium (5) and in this medium can be induced by hemin and butyric acid to synthesize hemoglobins (6).

We have investigated in more detail the minimal requirements of mouse and human erythroleukemia cell lines for growth in synthetic medium and whether other factors could improve the growth of the cells to densities comparable to those obtained using medium supplemented with 10 to 15% fetal bovine serum. Our results indicate that it is possible to continuously grow murine and human erythroleukemia cells in RPMI 1640, supplemented only with transferrin and selenium dioxide. The addition of bovine serum albumin (0.5 to 1 mg/ml) allows cells of the human line to grow to a density comparable to that obtained in serum-containing medium. We have also investigated the characteristics of the differentiation phenotype of the cells grown for extensive lengths of time in synthetic medium and their response to certain inducers of differentiation.

The inducers of hemoglobin synthesis in serum-containing medium which we have tested can also induce the appearance of hemoglobin-containing cells in synthetic medium. However, the percentage of benzidine-positive cells and the amount of hemoglobin synthesized are less than that observed when cells are grown in serum-containing medium. The process of spontaneous differentiation observed in cultures of cells grown with serum also occurs in cells growing in synthetic medium, indicating that expression of the differentiation program is controlled through intracellular mechanisms but that serum factors may modulate the extent of differentiation.

MATERIALS AND METHODS

Cell Lines. Friend erythroleukemia cells (GM86; clone 745 of DBA/2 mouse origin) (10) and GM79 clone 3TCI 12 of DDD mouse origin (25), were purchased from the Institute of Medical Research (Camden, New Jersey).

1 Supported by Grants CA-10815 and CA-24273 from the National Cancer Institute and by the W. W. Smith Memorial Fund.

2 The abbreviation used is: RPMI 1640, Roswell Park Memorial Institute Medium 1640; BSA, bovine serum albumin.
cells, a spontaneously differentiating clone of Friend cells, have been a gift of Paul Harrison (Beatson Institute, Glasgow, Scotland). D1 CI 7 cells, a spontaneously differentiating clone of Friend cells, have been described previously (26). We selected the human cell line K562(S) for its responsiveness to hemin and butyric acid induction of embryonal hemoglobin synthesis (6).

Medium. Three types of medium were used throughout the experiments reported here. Control cultures were grown in RPMI 1640 (No. 12-602-49; Flow Laboratories, Rockville, Md.) supplemented with 2 mM glutamine, 100 μg streptomycin, 100 units penicillin, and 15% fetal bovine serum (Flow Laboratories). The minimal synthetic medium consisted of RPMI 1640 supplemented with 2 mM glutamine, 100 μg streptomycin, 100 units penicillin, transferrin (5 μg/ml), 3 × 10⁻⁸ M selenium dioxide and further buffered with sodium bicarbonate and 15 mM 4-(2-hydroxyethyl)-1-piperazineethanesulfonic acid to pH 7.2. The high-density synthetic medium was made by adding albumin (0.5 to 1 mg/ml) to the minimal synthetic medium.

Human transferrin (Sigma Chemical Co., St. Louis, Mo.) was dissolved in phosphate-buffered saline at a final concentration of 5 mg/ml in 0.5-ml aliquots and stored at −20°C. 4-(2-Hydroxyethyl)-1-piperazineethanesulfonic acid (1.5 M; pH 7.2) (Sigma) was stored at −20°C. Selenium dioxide (3 × 10⁻⁸ M) (J. T. Baker Chemical Co., Phillipsburg, N. J.) was kept at 4°C. Bovine serum albumin charcoal extract (Collaborative Research, Inc., Waltham, Mass.) was reconstituted in water at a final concentration of 100 mg/ml, distributed in 2.5-ml aliquots, and stored at −20°C. The medium was prepared and used within 1 week after addition of various other components. Cells were incubated in a 5% CO₂:95% air humidified atmosphere at 37°C and refed every 3 to 4 days, seeding them at 0.5 to 1.5 × 10⁵ cells/ml. Cells were counted in a counter chamber. Viability was determined by trypan blue dye exclusion.

Induction of Hemoglobin Synthesis. Cells were induced to differentiate in serum-free medium by incubating them for 4 to 6 days in dimethyl sulfoxide (10), hexamethylene bisacetamide (24), hypoxanthine (14), or butyric acid (17) at the concentrations specified in the text. Cells were seeded at 0.5 × 10⁵/ml, and inducing agents were added 24 hr later. Benzidine-positive cells were determined as described by Orkin et al. (21). Hemoglobin was quantitated spectrophotometrically and characterized by electrophoresis on Cellogel as described previously (6).

Other Chemicals. Dimethyl sulfoxide was from Fisher Scientific Co. (Fair Lawn, N. J.), hypoxanthine from Sigma, and butyric acid from Aldrich Chemical Company, Inc. (Milwaukee, Wis.). Hexamethylene bisacetamide was synthesized as described by Germinario et al. (12). Appropriate dilutions were prepared fresh for each experiment. All other growth factors tested were from Collaborative Research.

RESULTS

Minimal Synthetic Medium. Human K562(S) cells and mouse erythroleukemia cells were seeded in Breitman’s serum-free synthetic medium supplemented by insulin, transferrin, and selenium dioxide (5, 6) at a concentration of 0.5 to 1.5 × 10⁵/ml and passed every 4 days. There was no appreciable lack of growth or period of adjustment of the cells during the transfer procedure. After 5 or more passages in this medium, the optimal concentration ranges for each of the 3 agents supplementing the medium were determined. As shown in Chart 1A, the reduction of transferrin immediately inhibited the growth of clone 745 murine erythroleukemia cells and of human erythroleukemia K562(S) cells. Both cell types grown in selenium dioxide-free medium grew well during the first passage (Chart 1B), but clone 745 cells stopped growing during the second. K562(S) continued to grow in this medium only if the cells were diluted 1:2 for the next 4 to 5 passages. After this adaptation period, K562(S) grew in selenium dioxide-free medium at their normal rate. Omitting insulin or increasing its concentration in the medium for any length of time did not affect the growth of the cell lines. Similar requirements were also observed for mouse erythroleukemia clones 707 and D1 Cl 7 of DBA/2 origin and for clone 3TCl 12 of DDD origin.

The data in Table 1 show the cell saturation density and doubling time of clone 745 and K562(S) cells grown in different media. After 4 days in culture, the final cell densities of K562(S) grown in serum-containing medium and in minimal synthetic medium were essentially the same, and the doubling time was only slightly longer in the minimal medium. By contrast, the cell density of GM86 grown in the minimal medium was approximately one-half that seen in serum-containing medium, and the doubling time was about twice as long.

High-Density Synthetic Medium. Since the saturation density of the erythroleukemia cells grown in synthetic medium was less than that observed using the serum-containing medium, we investigated whether the addition of other factors to the medium would result in a cell concentration comparable to that observed with serum. Hydrocortisone, dihydrotestosterone, estradiol, thyrotropin-releasing hormone, glucagon, progesterone, luteinizing hormone-releasing hormone, thyroid stimulating hormone, and follicle-stimulating hormone in a wide

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**Synthetic Media for Erythroleukemia Cells**

**Chart 1.** Growth and dose response curves of K562(S) cells and GM86 cells in minimal synthetic medium containing (a) constant amount of selenium dioxide (sel. diox.) (3 × 10⁻⁸ M) and various concentrations of transferrin; (b) various concentrations of selenium dioxide and constant amounts of transferrin (5 μg/ml). Cells initially seeded at 0.5 × 10⁵/ml were counted on Day 4.

- ○, K562(S); ▲, GM86.

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range of concentrations were found to be ineffective. As shown in Table 1, the addition of bovine serum albumin resulted in either a further growth of the K562(S) human erythroleukemia cells to densities higher than those observed in serum-containing medium or a decreased doubling time. The optimal concentration of albumin ranged from 500 μg/ml to 3 mg/ml. Addition of albumin to the minimal synthetic medium have high percentages (10 to 40%) of benzidine-positive cells (6, 26). When mouse erythroleukemic D1 Cl 7 and K562(S) cells were maintained in synthetic medium, a range of 5 to 30% of benzidine-positive cells was observed at all times. A higher percentage of benzidine-positive cells resulted if the culture was passed every 5 days rather than every 3 or 4 days, suggesting that the growth phases of the cell lines and possibly reduced frequency of cell divisions allowed the accumulation of benzidine-positive cells in the population (Table 2). The base level of spontaneous differentiation was only 2 to 3% when cells were never allowed to pass the maximal density of 5 × 10^5/ml.

**Induction of Differentiation in Cells Growing in Synthetic Medium.** The effect of a number of compounds known to induce hemoglobin synthesis in cells grown in serum-containing medium was tested in cells growing in minimal synthetic medium. The inducers tested were found to be toxic when added at the doses used in control cultures growing in serum-containing medium. Therefore, the optimal conditions of induction in minimal synthetic medium were determined (Table 3). All the inducers tested were effective in inducing differentiation of both Friend erythroleukemia cells and human erythroleukemia cells, although the percentage of benzidine-positive cells and the amount of hemoglobin per cell were less than that observed in control cultures grown in serum-containing medium. These findings suggest that serum contains other factors that enhance the inducibility of the cells and the response of a high percentage of cells to differentiation. The optimal concentrations of the inducers were lower than those for induction of cells in the presence of serum, although inducers at these concentrations were almost entirely inactive in serum-containing medium. The hemoglobins synthesized by cells growing in synthetic medium were analyzed by electrophoresis and found to be identical to those observed when cells were grown in serum-containing medium (data not shown).

**DISCUSSION**

Most of the minimal growth media that have been described share a common requirement for transferrin and insulin, with further requirements for other hormones depending on the cell type (4, 5, 7, 28). The minimal medium that supports the growth of human promyelocytic leukemia cells (HL-60) (5) contains insulin and transferrin. The minimal growth medium that we have described here for human and murine erythroleukemia cell lines requires only the presence of transferrin and selenium dioxide. In this synthetic medium, mouse erythroleukemia cells

**Table 1**

<table>
<thead>
<tr>
<th>Cell line</th>
<th>K562(S)</th>
<th>GM86</th>
</tr>
</thead>
<tbody>
<tr>
<td>Medium</td>
<td>Cell saturation density (×10^5/ml)</td>
<td>Doubling time (hr)</td>
</tr>
<tr>
<td>Control cultures (serum-containing medium)</td>
<td>1.24</td>
<td>36</td>
</tr>
<tr>
<td>Minimal synthetic medium</td>
<td>1.12</td>
<td>43</td>
</tr>
<tr>
<td>Synthetic medium supplemented with albumin</td>
<td>1.6</td>
<td>35</td>
</tr>
</tbody>
</table>

*a Viable cells (1.5 × 10^5) were seeded at time 0, and maximum saturation density was reached after 4 to 5 days.

**Table 2**

<table>
<thead>
<tr>
<th>Day</th>
<th>GM86</th>
<th>K562(S)</th>
<th>D1 Cl 7</th>
</tr>
</thead>
<tbody>
<tr>
<td></td>
<td>% of benzidine-positive cells (%)</td>
<td></td>
<td></td>
</tr>
<tr>
<td>1</td>
<td>0.50</td>
<td>8</td>
<td>1</td>
</tr>
<tr>
<td>2</td>
<td>ND^b</td>
<td>10</td>
<td>5</td>
</tr>
<tr>
<td>3</td>
<td>ND</td>
<td>18</td>
<td>ND</td>
</tr>
<tr>
<td>4</td>
<td>1</td>
<td>19</td>
<td>ND</td>
</tr>
<tr>
<td>5</td>
<td>1</td>
<td>26</td>
<td>34</td>
</tr>
</tbody>
</table>

*a Cells were seeded at 0.5 × 10^5/ml, and benzidine staining was done on each of 5 days.

**Table 3**

Optimal conditions for induction of differentiation for murine and human erythroleukemic cell lines grown in minimal synthetic medium

The values of B^+ cells in untreated controls are those reported in Table 2.

<table>
<thead>
<tr>
<th>Cell line</th>
<th>Butyric acid</th>
<th>Hexamethylene bisacetamide</th>
<th>Hypoxanthine</th>
<th>Dimethyl sulfoxide</th>
</tr>
</thead>
<tbody>
<tr>
<td>Dose (mM)</td>
<td>Day</td>
<td>B^+ (%)</td>
<td>Hb/cell (pg/cell)</td>
<td>Dose (mM)</td>
</tr>
<tr>
<td>K562(S)</td>
<td>0.4^a</td>
<td>4</td>
<td>64</td>
<td>1.0^b</td>
</tr>
</tbody>
</table>

*a Benzidine staining and amount of hemoglobin were determined for each of 5 days.

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from 3 sources (clone 745 and its subclone D1 Cl 7, clone 707, and 3TC12) and human erythroleukemia cells grew well for a sufficiently large number of passages (up to 70); this suggests that infinite growth is likely. However, in this medium, mouse cell lines did not reach the saturation density typically observed when cells are grown in serum-containing medium. When this minimal synthetic medium was supplemented with albumin, however, the doubling time was shorter in both cases and the human cells grew to a higher density than that obtained when grown in medium containing 15% fetal bovine serum. This higher cell density is due to a more rapid doubling time and to prolonged survival of the culture 3 days after seeding.

The presence of selenium dioxide was essential for the growth of the mouse erythroid cells. For the human cell line, selenium dioxide was not essential, but cells underwent an adaptation period for the first 4 to 5 passages after withdrawal of the chemical. Selenium dioxide is not essential for growth of myeloid cells (5) but is reported to be required in other systems (13, 20). Ebert and Malinin (8) have shown selenium dioxide to be an inducer of erythroid differentiation, however, only at concentrations that inhibit cellular growth.

In our initial studies on response to inducing agents, we used the minimal synthetic medium rather than the high-density medium containing albumin, inasmuch as albumin is known to be a protein carrier that binds small molecules (4) and thus cannot be easily quantitated. Albumin-containing media are appropriate, however, in studies involving RNA turnover and late events in terminal differentiation since high concentrations of albumin favor the optimal morphological differentiation of Friend cells (27). The optimal concentration of inducers for cultures of cells grown in medium with serum was found to be toxic for cultures growing in synthetic medium, and slightly lower doses were used to test the response of cells in synthetic medium. The differentiative response of cells growing in synthetic medium to dimethyl sulfoxide treatment is weaker than that in serum-containing medium. Kluge et al. (16) and Gazitt (11) made similar observations. Because the presence of serum caused the cells in our experiments to synthesize 3 to 5 times more hemoglobin when inducers were added, it is quite likely that the differentiation process of erythroleukemic cells is mediated by the chemical agents but is also modulated by other factors present in serum. In support of this hypothesis is the finding of Gazitt (11) that addition of L-ornithine, and our finding that addition of bovine serum albumin, insulin, or both partially enhances (from 10 to 50%, depending on the inducer used) the number of benzidine-positive cells and the amount of hemoglobin synthesized by induced cells in serum-free medium. The inducers are active in synthetic medium at doses inactive in serum-containing medium. Our observations on reduced differentiative response in synthetic medium are at variance with those of Breitman et al. (5) who have reported that the level of spontaneous and induced differentiation for the human promyelocytic leukemia HL60 cell line is the same in serum-containing medium and synthetic medium.

Development of serum-free medium for growing established cell lines has been the subject of several recent investigations (4). There are several disadvantages in using serum in the culture of cells, including the present high cost of fetal bovine serum, the possibility of Mycoplasma infections due to improperly assayed batches of serum, and variability of activity of different batches of serum. The most important disadvantage in using serum, however, lies in the heterogeneity of factors that it contains. Some of these factors are necessary for growth, some are needed for differentiation, while still others are toxic for particular cell populations. The synthetic medium that we have described eliminates these variables and permits the cells to grow for a virtually unlimited number of passages with a doubling time comparable to that obtained by growth in serum, i.e., when cells are growing at their maximum rate. This medium can thus reliably and completely replace serum-containing medium. It will also allow a more accurate identification of the extracellular factors involved in the expression of the full program of erythroid differentiation.

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REFERENCES

1. Abraham, J., and Rovera, G. Inducers and inhibitors of leukemic cell differ-
entiation in culture. In: G. V. R. Born, O. Eichler, A. Farah, H. Herken, and
A. D. Welch (eds.), Handbook of Experimental Pharmacology. Berlin: Sprin-
ger-Verlag, in press, 1981.
differentiation in the human leukemia cell line K562. Nature (Lond.), 278:
insulin and transferrin supports growth and differentiation of the human
6. Cioè, L., McNab, A., Hubbell, H. R., Meo, P., Curtis, P., and Rovera, G.
Differential expression of the globin genes in human leukemia K562(5) cells
induced to differentiate by hemin or butyric acid. Cancer Res., 41: 237–
243, 1981.
in serum-free medium: lack of involvement of the cyclic AMP pathway in long-
8. Ebert, P. S., and Malinin, G. I. Induction of erythroid differentiation in Friend
murine erythroleukemic cells by inorganic selenium compounds. Biochem.
9. Friend, C., Preisler, H. D., and Scher, W. Studies on the control of differ-
etiation of murine virus-induced erythroleukemic cells. Current Topics
10. Friend, C., Scher, W., Holland, J. G., and Sato, T. Hemoglobin synthesis in
murine virus-induced leukemia cells. In vitro stimulation of erythroid differ-
1971.
11. Gazitt, Y. Comparative study of two groups of inducers of Friend erythroleu-
kenia cell differentiation in a chemically defined medium. Cancer Res., 41:
12. Germinario, R. J., Kleiman, L., Peters, S., and Oliveira, M. Decreased deoxy-
D-glucose transport in Friend cells during exposure to inducers of erythroid
transferin, albumin and lectin in haemopoietic cell cultures. Nature
A. D. Welch (eds.). Handbook of Experimental Pharmacology. Berlin: Sprin-
ger-Verlag, in press, 1981.
synthesis in Friend erythroleukaemia mouse cells in protein and lipid-free
17. Leder, A., and Leder, P. Butyric acid—a potent inducer of erythroid differ-
18. Llozio, C. B., and Llozio, B. B. Human chronic myelogenous leukemia cell-
Growth and Differentiation of Human and Murine Erythroleukemia Cell Lines in Serum-free Synthetic Medium

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