Effect of Hexamethylene Bisacetamide on the Commitment to Differentiation of Murine Erythroleukemia Cells

Eitan Fibach, Roberta C. Reuben, Richard A. Rifkind, and Paul A. Marks

Departments of Medicine and Human Genetics and Development, and the Cancer Research Center, Columbia University, New York, New York 10032

SUMMARY

Friend virus-transformed murine erythroleukemia cells express the program of erythropoietic differentiation under the influence of the previously described, potent inducing agent, hexamethylene bisacetamide. Commitment to differentiation, defined as the ability to continue the processes of differentiation in the absence of inducer, has been examined at the single-cell level, with a combination of suspension and cell-cloning techniques. Recruitment of committed cells is shown to occur prior to the detectable accumulation of hemoglobin or the appearance of morphological changes characteristic of erythroid maturation. The stability of the commitment of murine erythroleukemia cells to differentiation is found to be dependent upon both the concentration of hexamethylene bisacetamide and the duration of exposure to the inducing agent. Under conditions less than optimal for induction, a single cell can give rise to a colony containing both differentiated and undifferentiated cells. On the basis of these findings, it is suggested that fully stabilized differentiation, in addition to the previously demonstrated requirement for the inducing agent to be present during a cell-cycle S phase, involves subsequent stabilizing event(s) caused by a direct or indirect action of the inducing agent.

INTRODUCTION

MELC* line 745A, developed by Charlotte Friend, displays a low percentage (<1%) of spontaneous erythroid differentiation (3). The addition of an inducing agent such as dimethyl sulfoxide or HMBA to the culture medium can cause up to 100% of these cells to express the program of erythroid differentiation, including characteristic morphological changes and synthesis of hemoglobin (4, 8). There is evidence that dimethyl sulfoxide must be present during DNA synthesis to induce differentiation of MELC (5, 6). This study was designed to define further the relationship between exposure of MELC to HMBA and the transition to erythroid differentiation.

In order to study the kinetics of recruitment to differentiation induced by HMBA, a technique for assay of the commitment of individual cells to differentiate has been developed. In this assay, following suspension culture with HMBA, cells are cloned in a semisolid medium in the absence of the inducer, and the emergent clones are scored by the benzidine reaction for erythroid differentiation. It was found that the proportion of cells in a culture that is committed to differentiate is dependent upon both the concentration of HMBA and the duration of exposure to the inducing agent. HMBA-induced erythroid differentiation is associated with a limitation in proliferative capacity, as is characteristic of normal erythropoiesis. Under conditions less than optimal for induction of differentiation, induction to differentiation is not fully stabilized. Under these conditions, a single cell can give rise to a colony containing both differentiated and undifferentiated cells. Stabilized differentiation is essentially complete when cells are exposed to 5 mM HMBA for 50 hr.

MATERIALS AND METHODS

MELC, strain 745A, were kindly provided by Dr. Charlotte Friend. Our subclone, designated DS19, has been maintained in suspension culture as described elsewhere (7, 9). Unless otherwise noted, cultures were inoculated at a density of 10^5 cells/ml from 3d cultures. Cell density was determined by Coulter counter.

Cells were cloned in semisolid medium composed of 1.44% (w/v) methylcellulose (400 centipoise; Fisher Scientific Co., Pittsburg, Pa.) in Dulbecco’s modified Eagle’s medium (Grand Island Biological Co., Grand Island, N. Y.) supplemented with 15% (v/v) fetal bovine serum (9). Cells were suspended at 5 × 10^4 cells/ml in semisolid medium and dispensed in 1-ml aliquots into 35-mm plastic Petri dishes ( Falcon Plastics, Oxnard, Calif.). Cultures were examined immediately after inoculation to ensure that the cells were distributed singly, not as clusters.

Cells from liquid suspension cultures were stained for hemoglobin content either in suspension by the benzidine reaction in acetic acid (8), in which case the proportion of blue-stained cells was scored, or by depositing the cells on a glass slide (cytocentrifuge; Shandon Southern Instruments, Inc., Sewickly, Pa.) fixing in methanol and staining with the alkaline benzidine-Wright-Giemsa reaction (9), in which case the proportion of orange-stained cells was scored. The 2 methods give comparable results, although the former is somewhat more sensitive to low levels of

1 Supported in part by grants and contracts from the National Institutes of Health (GM-14552, CA-13696, CA-18316, NO1-CB-4-4008, NO1-CP-6-1008) and the National Science Foundation (NSF-PCM-75-08696).
2 Fellow of the Schultz Foundation.
3 To whom requests for reprints should be addressed.
4 The abbreviations used are: HMBA, hexamethylene bisacetamide; MELC, murine erythroleukemia cells.

Received August 20, 1976; accepted November 3, 1976.
hemoglobin. Colonies in semisolid medium were stained for hemoglobin-containing cells in situ with the benzidine reagent in acetic acid (1). Individual colonies were scored for the presence of benzidine-reactive cells by examination with an inverted microscope.

DNA synthesis was scored by incubation of cells with tritiated thymidine (0.3 μCi of [methyl-3H]thymidine; specific activity, 20 Ci/mole [New England Nuclear, Boston, Mass.] added to the culture prior to inoculation with cells. The proportion of cells labeled with tritium was assayed by radioautography; cells were scored as labeled if there were 3 or more grains per cell, where background was less than 1 grain/average cell area (2).

HMBA was prepared as previously described (8).

RESULTS

Erythroid Differentiation in Suspension Cultures. Benzidine-reactive cells begin to accumulate after 50 hr in MELC cultures grown in media containing 5 mM HMBA. Thereafter, there is a linear increase in the proportion of benzidine-reactive cells, reaching 90% or more by 96 hr (Chart 1). During the period from 50 to 120 hr, the differentiating cells display increasing intensity of staining with benzidine, as well as the morphological changes characteristic of erythropoiesis, including a decrease in cell size, nuclear heteropycnsis and, occasionally, nuclear extrusion. Cell division can continue during these morphogenetic and biochemical events, as evidenced by some increase in cell density and the presence of benzidine-reactive mitotic cells.

Relationship between Duration of Exposure to HMBA and Commitment to Differentiate. Experiments were performed to determine whether the presence of inducer is required continuously throughout the process of erythroid differentiation or whether there is a stage at which MELC become committed to differentiate despite removal of the agent from the culture medium. Cells were cultured with 5 mM HMBA for varying items (5 to 100 hr) prior to transfer to fresh suspension medium without HMBA (Chart 1). Induction of differentiation in these "transfer-out" studies was assayed by determining the proportion of cells which become benzidine reactive after a total of 120 hr of culture. Under these conditions, commitment to differentiation above the control (no HMBA) level (about 0.5%) was detected after 24 hr of exposure to HMBA. Cells committed to differentiate are detected, by this technique, at least 24 hr before they display hemoglobin accumulation by the acid benzidine reaction. The proportion of committed cells increases linearly with time of exposure to HMBA from 24 to 80 hr. MELC committed to differentiate after a short period (24 to 48 hr) of exposure to HMBA become equally mature, by the criteria of morphology and the benzidine reaction, as MELC exposed continuously to HMBA for 120 hr. These results suggest that there is a stage in the process of differentiation, prior to the accumulation of hemoglobin, which we term "commitment," after which MELC can continue the developmental process in the absence of inducer in the culture medium.

Effect of HMBA on Commitment Assayed by Cloning. Suspension culture studies cannot adequately quantitate commitment to differentiation at the single cell level because of the possibility of differential growth rates for committed and uncommitted MELC. To determine the kinetics of commitment to differentiation and the capacity for replication of single cells following culture with HMBA at various concentrations and for various times, transfer-out was accomplished by transfer, after washing the cells, to a semisolid medium without inducer (Chart 2). Under these conditions, both induced and uninduced cells produce colonies that can be scored for differentiation by staining the culture plates with the benzidine reagents on the 5th day after initiation of the suspension cultures. Control cells, grown in suspension culture without HMBA for 24 to 72 hr, then transferred to semisolid medium, consistently yielded less than 3% of colonies containing benzidine-reactive cells. Over 90% of cells inoculated can be detected on Day 5 as colonies or small clusters, in experiments using from 0 to 5.0 mM HMBA. Commitment was first detected in cells exposed to 5 mM HMBA for 16 h (Chart 2). The percentage of colonies containing benzidine-reactive cells increased linearly with duration of exposure to inducer, reaching 100% by 50 hr. To test whether cells inoculated while in log-phase of growth would display a shorter lag to commitment, suspension cultures were initiated with 1-day (log phase) rather than 3-day (stationary phase) cells. Whereas cultures inoculated with stationary phase cells show a distinct lag before initiating log phase growth (5, 7), cultures inoculated with log phase cells do not display a significant growth lag. Under these conditions, committed cells were first detected after 12 to 13 hr of exposure to HMBA; the rate of increase in proportion of committed cells was equal to that of the stationary phase cultures, but virtually 100% induction was achieved about 4 to 5 hr earlier (Chart 2). These observations suggest that cells that are in log phase of growth are susceptible to commitment earlier than stationary phase cells.

Effect of HMBA Concentration on the Rate of Commitment. The rate at which cells exposed to HMBA in suspen-
Heterogeneity of colonies derived from MELC cultured with various concentrations of HMBA and for various times. Cells were exposed in suspension culture to various concentrations of HMBA for 53 hr (Part 1) or to 5 mM HMBA for various periods before cloning (Part 2) in semisolid medium in the absence of the inducer. Colonies were scored, 5 days from initiation of the experiments, by benzidine staining.

**Chart 2.** Effect of duration of exposure to 5 mM HMBA on the recruitment of MELC committed to differentiate. Commitment was assayed as in Chart 2 after 24 hr (●) and after 53 hr (○) of exposure to the concentrations of HMBA indicated. Cloning efficiency of cells exposed to HMBA for 24 hr is expressed as the number of colonies present on the plate divided by the number of cells that were inoculated in the medium multiplied by 100 (△).

**Chart 3.** Effect of concentration of HMBA on the recruitment of MELC committed to differentiate. Commitment was assayed as in Chart 2 after 24 hr (●) and after 53 hr (○) of exposure to the concentrations of HMBA indicated. Cloning efficiency of cells exposed to HMBA for 24 hr is expressed as the number of colonies present on the plate divided by the number of cells that were inoculated in the medium multiplied by 100 (△).

**Table 1**

<table>
<thead>
<tr>
<th>HMBA (mM)</th>
<th>% undifferentiated colonies*</th>
<th>A. Uniformly benzidine reactive</th>
<th>B. Mixed colonies*</th>
<th>C. Proportion of mixed colonies*</th>
</tr>
</thead>
<tbody>
<tr>
<td>0</td>
<td>100</td>
<td>0</td>
<td>0</td>
<td>60</td>
</tr>
<tr>
<td>0.5</td>
<td>95</td>
<td>2</td>
<td>3</td>
<td>30</td>
</tr>
<tr>
<td>1.0</td>
<td>65</td>
<td>28</td>
<td>7</td>
<td>20</td>
</tr>
<tr>
<td>2.0</td>
<td>19</td>
<td>76</td>
<td>5</td>
<td>6</td>
</tr>
<tr>
<td>3.0</td>
<td>8</td>
<td>90</td>
<td>2</td>
<td>2</td>
</tr>
<tr>
<td>4.0</td>
<td>5</td>
<td>94</td>
<td>1</td>
<td>1</td>
</tr>
<tr>
<td>5.0</td>
<td>3</td>
<td>96</td>
<td>1</td>
<td>1</td>
</tr>
</tbody>
</table>

1. Studies with various concentrations of HMBA in precollonying culture for 53 hr

2. Studies with various times of precollonying culture with 5 mM HMBA

*The criterion for an undifferentiated colony is the absence of any benzidine-reactive cells.

*The criterion for a mixed colony is the presence of benzidine-reactive and benzidine-unreactive cells in the same colony.

C = \( \frac{A + B}{B} \times 100 \).

E. Fibach et al.
10^4 to 5 \times 10^3 \text{ cells/ml}; \text{ under these conditions, the proportion of mixed colonies was independent of inoculum concentration. These observations suggest that, under these conditions, a committed cell may give rise to both differentiated and undifferentiated progeny.}

The number of cells in a colony was related to the proportion of benzidine-reactive cells in the colony. Colonies without benzidine-reactive cells as well as mixed colonies continued to increase in size throughout the period of culture, which was up to 7 days. Uniformly benzidine-reactive colonies were smaller and generally showed no increase in size after the 4th day. For example, uniformly benzidine-reactive colonies derived from cells exposed to 5 mM HMBA for 24 hr grew to contain approximately 16 to 32 cells, while colonies derived from cells exposed for 48 hr contained predominantly 2 to 4 cells. These observations suggest that induction to differentiation is associated with a limitation in potential for cell division. This is consistent with the pattern of terminal differentiation characteristic of normal erythropoiesis (2).

**Relationship of DNA Synthesis to HMBA-induced Differentiation.** In previous studies (5, 6), it has been shown that dimethyl sulfoxide must be present during DNA synthesis and, possibly, during a portion of the cell cycle thereafter, in order to induce differentiation of MELC. If exposure to inducer during DNA synthesis is sufficient to commit a cell to differentiate, then the rate at which cells enter S phase of the cell cycle will equal the rate of induction to differentiation. Alternatively, if exposure to inducer during S phase is required but not sufficient for commitment, then the rate of entry into S will exceed the rate of induction. These alternatives were examined by scoring cells for DNA synthesis by [3H]thymidine uptake and radioautography during HMBA-induced differentiation. The rate of entry into S phase, as measured in this fashion, was greater than the rate of induction to differentiation, as measured by transfer-out from medium with HMBA to medium without inducer (Chart 1).

**DISCUSSION**

In the studies reported in this paper we have examined, at the cellular level, the commitment of MELC to differentiated erythroid cells producing hemoglobin. HMBA is a very efficient inducer, previously shown to commit essentially the entire population of MELC (line DS19, derived from Friend's 745A) to differentiate, under appropriate conditions in suspension culture (8). In the present studies, commitment to erythroid differentiation, defined as the ability to continue differentiation in the absence of inducer in the medium, was assayed by cloning cells in the absence of inducing agent, after exposure to HMBA in suspension culture at various concentrations and for various times. Colonies were scored for proliferative capacity (size of the colony) and production of hemoglobin (benzidine reaction). It was shown that recruitment of committed MELC is dependent on the concentration of HMBA and on the duration of exposure to the agent. Between 0.5 and 5.0 mM HMBA, the higher the concentration, the greater the proportion of cells recruited to differentiate. Between 12 and 53 hr, the longer the period of exposure, the greater the proportion of cells committed.

Previous observations from this laboratory (5) and elsewhere (6) indicate that DNA synthesis is required for the commitment of MELC to differentiate. The present studies suggest that DNA synthesis (passage through S phase of the cell cycle) is a necessary but not sufficient factor in this process of induced differentiation. Thus, inculating log phase cells eliminates the growth lag in culture and shortens the exposure to HMBA required for commitment, as would be expected if a cell cycle were required for commitment. However, the rate of entry of MELC into S phase is greater than the rate of recruitment of committed erythroblasts, suggesting that additional steps or events, with different kinetics, are required to achieve commitment of an erythroleukemic cell to differentiate.

Commitment to differentiation may not be irreversible under all conditions. Exposure of MELC to a suboptimal concentration of HMBA or for a shorter than optimal period prior to cloning in inducer-free medium can yield progeny from a single cell which include differentiated and undifferentiated cells. It may be speculated that a critical concentration of inducer or a product of inducer cell interaction must be maintained during events of the cell cycles of the target cell and its progeny that are critical to the stabilization of differentiation. Under suboptimal conditions of induction, some progeny display instability of induced differentiation and fail to express the program of erythropoietic development. Increasing the concentration of inducing agent or the duration of exposure achieves stabilized differentiation in an increasingly large proportion of cells. Under optimal conditions, virtually all MELC and their progeny are irreversibly committed to differentiate by the very efficient inducing agent, HMBA. It may further be proposed that inducing agents that are less efficient than HMBA (that is, that initiate differentiation in a smaller proportion of MELC) may be less efficient because they cannot achieve a fully stabilized differentiating state. As a working hypothesis, these studies suggest that commitment to differentiation may comprise a series of events, with different kinetics, including a triggering effect related in part to the S phase of the cell cycle and a subsequent stabilizing event required to render differentiation irreversible.

**REFERENCES**

E. Fibach et al.


Effect of Hexamethylene Bisacetamide on the Commitment to Differentiation of Murine Erythroleukemia Cells


Updated version  Access the most recent version of this article at: http://cancerres.aacrjournals.org/content/37/2/440

E-mail alerts  Sign up to receive free email-alerts related to this article or journal.

Reprints and Subscriptions  To order reprints of this article or to subscribe to the journal, contact the AACR Publications Department at pubs@aacr.org.

Permissions  To request permission to re-use all or part of this article, contact the AACR Publications Department at permissions@aacr.org.