Sensitivity to 5-Azacytidine of Blast Progenitors in Acute Myeloblastic Leukemia

By Chen Wang and E.A. McCulloch

In a previous study, we showed that the blast stem cells of acute myeloblastic leukemia (AML) were more sensitive to cytosine arabinoside (ara-C) when growing in suspension culture than during colony formation in methylcellulose. We suggested that the difference might be explained by considering the cellular mechanisms responsible for growth in suspension and colony formation. In the former, the clonogenic cells increase in number (self-renewal); in the latter, most of the divisions are terminal. The increased sensitivity to ara-C in suspension might then be attributed to its ability to inhibit self-renewal to a greater degree than cell division generally. A test of this hypothesis would be to compare the survival curves in suspension and in methylcellulose using a drug that spared or stimulated self-renewal. Such an agent is 5-azacytidine (5-aza) and has the additional advantage that its analogue, 6-azacytidine (6-aza) has no effect on self-renewal. The data supported the hypothesis, since clonogenic AML blasts were much more sensitive to 5-aza in suspension than in methylcellulose. The effect of 6-aza, while qualitatively similar, was much less marked. Controls showed that the difference in survival curves could not be explained on a kinetic basis or by the secretion of growth factors by 5-aza–treated cells. We suggest that a comparison of the effects of drugs in suspension and in methylcellulose may be useful in preclinical screening of putative anti-AML compounds.

SELF-RENEWAL, the process through which stem cells give rise to daughters with sustained proliferative potential, is essential for the maintenance of normal or malignant hematopoietic clones. The blast progenitors in acute myeloblastic leukemia (AML) have been shown to be capable of self-renewal. These malignant stem cells form colonies in methylcellulose-containing cultures when stimulated by appropriate growth factors. A minority of cells from blast colonies form new blast colonies on replating. Furthermore, clonogenic blast cells increase exponentially in suspension culture. Both these events can be measured quantitatively, and each may be considered a manifestation of blast self-renewal.

We suggested that self-renewal may be an important target for therapeutic agents used in the treatment of AML. First, there is a consistent association between high self-renewal capacity as measured by replating pooled blast colonies, (secondary plating efficiency, or PE2) and poor response to treatment. Second, we showed that cytosine arabinoside (ara-C), a drug included in most effective anti-AML regimens, decreases self-renewal of blast progenitors cultured in its presence. Recently, we found that ara-C is a more potent inhibitor of blast growth in suspension than of blast colony formation in methylcellulose. Moreover, preliminary data from a small number of patients provided evidence that the sensitivities of blasts measured in suspension were associated with outcome of treatment with ara-C, whereas similar survival curves obtained with the methylcellulose assay showed no such correlation.

We postulated that ara-C appeared to be more toxic to growth of cells in suspension than to colony formation in methylcellulose because self-renewal was the predominant cellular event in the former, whereas colony formation reflected principally postdeterministic divisions. A test of this hypothesis would be to measure survival curves under both conditions, using an agent that increased self-renewal. The drug 5-azacytidine (5-aza) is an obvious candidate; not only does exposure to it increase self-renewal, but a control compound also exists. The analogue 6-azacytidine (6-aza) is cytotoxic but, unlike 5-aza, does not affect self-renewal.

This article reports the measurement of 5-aza survival curves for AML blast cells exposed to the drug either in suspension or in methylcellulose. As predicted, growth in suspension was less sensitive to 5-aza than was colony formation in methylcellulose.

MATERIALS AND METHODS

Leukemic cells. Heparinized peripheral blood was obtained from 20 patients with AML. (French-American-British, FAB, classification M1 through M5) at the time of diagnosis or at relapse. The diagnosis was based on morphological and clinical criteria regularly used in clinical trials of patients with AML at the Ontario Cancer Institute. Only patients with a high percentage of blast cells in the peripheral blood were included in the study.

Preparations of blast cells free of contaminating T lymphocytes were obtained as described previously, using two Ficoll-Hypaque density-gradient centrifugations (P = 1.007), the second following the formation of sheep-erythrocyte rosettes. The cells were either placed in culture at once or preserved in liquid nitrogen in the presence of 10% dimethylsulfoxide (DMSO) and 50% fetal calf serum (FCS). In some experiments, cells were tested after being maintained for up to 3 months in suspension culture.

Assay for clonogenic blast cells. The assay for clonogenic blast cells was as described previously with the exception that media conditioned by the bladder cancer cell line HTB9 (HTB9-CM) was used as a source of growth factor. Blast populations were cultured in 35-mm Lux dishes (Miles Laboratories, Naperville, IL.) in a medium consisting of α-minimal essential medium (MEM), 20% FCS (growth medium) and 10% HTB9-CM with 0.8% methylcellulose. The cultures were incubated for 7 days in a humidified atmosphere in the presence of 5% CO2 in air at 37 °C, after which...
time colonies containing >20 cells were counted, using an inverted microscope.

Measurement of the PE2. The secondary plating efficiency (PE2) was measured using the pooled colony technique described previously. The cells were harvested from primary colonies, pooled, washed and replated in microwells at a cell density of 10⁶ per well with media of the same composition used for primary cultures. Colonies with >20 cells were counted after 7-day incubation. PE2 was expressed as colonies per 10⁶ cells plated.

Suspension culture method. Blast cells were cultured in suspension as described previously. As in the clonogenic assay, HTB9-CM was used rather than phytohemagglutinin-leukocyte conditioned medium (PHA-LCM) as a source of growth factor. The cells were suspended in growth medium at a concentration of 10⁶/mL in the presence of 10% HTB9-CM. After varying times of incubation, the cells were harvested and counted. Usually the cell numbers recovered at this point in the control groups were greater than the 10⁶ cells/mL used at the beginning of the culture: the suspensions were washed and replated in methylcellulose for blast colony formation, as described earlier. This procedure provided a measurement of the number of clonogenic cells per dish recovered at each point of time in suspension culture. The calculation consisted of multiplying the number of cells recovered per dish (as determined before washing) by the plating efficiency in methylcellulose. In some instances, cells recovered from suspension culture were washed, resuspended at 10⁶/mL, and recultured in suspension.

Exposure to drugs. 5-Aza was obtained from Sigma Chemical, St Louis, and 6-aza (P-L Biochemicals, Milwaukee). The drugs were dissolved in phosphate-buffered saline (0.02 mol/L Na₂HPO₄; 0.02 mol/L NaH₂PO₄, pH 6.5; 0.42 mol/L NaCl) just before use. Adriamycin was obtained from Upjohn, Don Mills, Canada. Adriamycin was modified to exclude ribonucleosides and deoxyribonucleosides and buffered with HEPES. The survival curves were calculated by linear regression. This procedure yielded two curves for the experiment with adriamycin, but SD of the points overlap and the two curves are not different.

Results

Figure 1 contains typical survival curves for blast stem cells maintained for 2 weeks in suspension culture and then exposed for 7 days to 5-aza, adriamycin, or ara-C, either in suspension or in methylcellulose. It is evident from Fig 1 that the comparisons of the survival curves in suspension and methylcellulose differed for the three drugs. As in previous studies, the adriamycin survival curves in suspension and methylcellulose were indistinguishable; in contrast, ara-C appeared more toxic to cells in suspension than to the same population in methylcellulose. The reverse was found for 5-aza; for this drug, the D₀ value in suspension was much greater than the D₀ in methylcellulose. Similar data were obtained when cells were exposed to the three drugs after 3 months in suspension culture. Table 1 contains the data from 20 experiments comparing 5-aza survival curves for blasts obtained either freshly or after cryopreservation, as well as the D₀ values in suspension and methylcellulose. Table 1 also shows the French-American-British (FAB) classification, the blasts in the peripheral blood, and the PE2 values for each patient. It is evident that the D₀ values in suspension were always greater than the D₀ values in methylcellulose (P < .00001); furthermore, there was no correlation between the two D₀ measurements (r = -.217). In agreement with previous findings, colonies in methylcellulose surviving exposure to 5-aza usually had increased values of PE2. In 4 of the 20 examples, however, decreases were seen.

As in previous experiments, treatment with 5-aza did not stimulate secondary colony formation if it was absent in controls (two examples in Table 1).

Controls. A number of controls were required to establish the validity of the differences in D₀ illustrated in Table 1. First, the construction of survival curves depends on a linear relationship between the number of cells placed in culture and the growth observed at the end of the assay. Linearity has been shown for methylcellulose cultures; for the suspension assay, a linear relationship exists between the number of nucleated cells used to initiate the cultures and the number of clonogenic cells recovered from each dish after 7 days. It was still possible, however, that treatment with 5-aza might change this relationship. Figure 2 shows the results of testing for linearity both in control suspension cultures and in cultures containing 5-aza; linearity was maintained under both conditions.

<table>
<thead>
<tr>
<th>5-Aza (,Mol)</th>
<th>Adriamycin (10⁻³ g/L)</th>
<th>Ara-C (,Mol)</th>
</tr>
</thead>
<tbody>
<tr>
<td>0</td>
<td>0.05</td>
<td>0.25</td>
</tr>
<tr>
<td>0.5</td>
<td>0.1</td>
<td>0.5</td>
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Fig 1. Survival curves for cells exposed to 5-azacytidine (5-aza) (A), adriamycin (B), or cytosine arabinoside (ara-C) (C) either in methylcellulose or in suspension. In the former instance, the drug was included in the cultures used for colony formation. In the latter instance, the drug was present during 7 days of suspension culture; the cells were then washed and tested for colony formation in methylcellulose. The number of clonogenic cells in each culture was calculated from the number of nucleated cells recovered from the 7-day cultures and plating efficiency (described in text). The cells used for this experiment were maintained in suspension culture for 2 weeks. Error bars show SD. Survival curves were drawn by linear regression. This procedure yielded two curves for the experiment with adriamycin, but SD of the points overlap and the two curves are not different.
Table 1. Clinical Characteristics and Response of Blast Cells to 5-Aza of Patients With AML

<table>
<thead>
<tr>
<th>Patient No.</th>
<th>Diagnosis (FAB)</th>
<th>Peripheral Blood</th>
<th>M.C.</th>
<th>PE2*</th>
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<tr>
<td>1</td>
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<td>51</td>
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<tr>
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<td>11.5 121 203 78</td>
<td>1.0 2.6</td>
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<tr>
<td>3</td>
<td>M5</td>
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<td>1.04</td>
<td>324 362</td>
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<tr>
<td>4</td>
<td>M5</td>
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<td>1.1 3.12</td>
<td>8.6 14</td>
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<tr>
<td>5</td>
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<td>11.9 271 39 77</td>
<td>1.2 3.2 1439 1749</td>
<td></td>
</tr>
<tr>
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<td>0.7 5.2</td>
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</tr>
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<td>1.2 2.8</td>
<td>10 24</td>
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<tr>
<td>8</td>
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<td>0.9 3.9 5.3 11</td>
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</tr>
<tr>
<td>9</td>
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<td>NA 2.2</td>
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<td>M5</td>
<td>8.4 62 75 19</td>
<td>1.1 2.9 168 487</td>
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<td>1.0 2.0 42 58</td>
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</tr>
<tr>
<td>20</td>
<td>M2</td>
<td>11.0 35 41 85</td>
<td>0.95 1.1 356 420</td>
<td></td>
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</table>

5-Aza, 5-azacytidine; FAB, French-American-British classification; D0, dose required to reduce survival to 10% of control; PE2, secondary plating efficiency; M.C., methylcellulose culture.

*PE2* values are the mean of four replicate microwells with 1 × 10⁶ cells from primary methylcellulose culture. The 5-aza group was at a concentration of 1.0 μmol/L.

Second, the outcome of suspension cultures is more dependent than colony formation on growth kinetics; in the latter, the number of cell divisions occurring during the assay period is reflected in colony size and does not enter into the analysis unless the number of cells in each colony is reduced below the counting criterion of 20 cells to a colony. For the suspension method, however, the number of doublings in 7 days contributes directly to the number of clonogenic cells recovered in each dish. Controls were necessary, therefore, to test the possibility that the apparent difference in the survival curves in suspension and methylcellulose could be explained by growth kinetics in suspension. Blast cells were grown in suspension in the presence or absence of 5-aza at a concentration of 10⁻⁶ mol/L, a concentration that is close to the D0 value in methylcellulose (Table 1). Two examples are illustrated in Fig 3. As anticipated, there was a sharp drop in clonogenic cells in each dish in the presence of drugs as compared with control. Suppression persisted for ~3 days and then growth resumed, with kinetics similar to the controls.

To exclude an early effect of 5-aza similar to that which occurred with ara-C, a detailed survival curve was prepared for cells maintained for >1 year in suspension and then exposed to 5-aza for 8 hours; the cells were then washed twice and either placed in suspension or plated in methylcellulose. The cells cultured in suspension were plated in methylcellulose after 7 days to obtain a survival curve in the usual manner. Figure 4 depicts the survival curves from this experiment, using the same cells, exposed to 5-aza briefly and then either plated directly in methylcellulose or maintained for 7 days in suspension. With this design, the reduction in growth in suspension was much less than that which occurred in methylcellulose.

Complete survival curves were also prepared for blast populations cultured for 3 days with or without 5-aza and then plated in methylcellulose to measure survival. Blasts exposed to 5-aza for 7 days in suspension or in methylcellulose were included in the same experiments as positive controls. Results from one of two similar experiments are shown in Fig 5. As in the curves prepared from cells exposed

![Fig 2. The linear relationship between number of nucleated cells added to suspension cultures and recovery of clonogenic cells after 7 days. Linearity is maintained in the presence of 5-aza (5-aza). Error bars show SD; lines were drawn by linear regression.](image-url)
to drug for 8 hours (Fig 4) or 7 days, the clonogenic cells exposed in suspension culture for 3 days were more resistant than in methylcellulose.

We also assessed the survival of the 5-aza structure. The drug was incubated in buffer without cells, using spectrophotometric absorption at 241 nm, as described in the Materials and Methods section. The drug was unstable, approaching undetectable levels after 3 days, a time consistent with the period of suppression of growth in culture.

As a third control, the effects of 6-aza were compared with those of 5-aza; survival curves were measured in seven experiments, both in suspension and methylcellulose. In agreement with the previous data, 6-aza was less toxic than 5-aza, using the clonogenic assay \( (P = .04) \). In contrast, in suspension, 6-aza was a more effective inhibitor than 5-aza \( (P = .04) \), although the \( D_{10} \) values in suspension for 6-aza were greater than the \( D_{10} \) values in methylcellulose \( (P = .004) \). For this subset of the data on blasts exposed to 5-aza, as for the whole data set, the \( D_{10} \) values for survival curves measured in suspension were much greater than those obtained using the methylcellulose assay \( (P = .007) \). Furthermore, the difference between survival curves in methylcellulose and in suspension for 5-aza was significantly greater than the differences for 6-aza \( (P = .011) \). Representative survival curves illustrating these features are shown in Fig 6.

**Growth factor production.** 5-Aza-treated blasts might show increased self-renewal as compared with controls if they were induced by the drug to secrete growth factors. Previously, we tested for this possibility, using cells that produced small amounts of growth factor; no increase was found following exposure to 5-aza. We extended these observations to cells that required HTB9-CM for growth and another blast population, representative of a rarer phenotype, in which good growth occurred in the absence of added HTB9-CM. For the former, it was necessary to add HTB9-CM to the cultures; therefore, controls were needed for the carryover of growth factors when the supernatants were tested for their effects on blast growth. This required titrations of HTB9-CM that had been incubated for 7 days in the presence of growth medium but without cells. For the population capable of sustained growth, these further controls were not required since these cells could be maintained in culture in the presence or absence of 5-aza without added growth factors. The results are given in Fig 7. All activity that occurred in cultures of cells requiring growth factor could be attributed to carryover of HTB9-CM; furthermore, no increase in growth-promoting ability was observed when
5-azacytidine and AML blast progenitors

Fig 7. Experiments designed to show whether blast populations secreted growth factors active on blast cells. The top hatched area is the 95% confidence interval for blast cells exposed to 10% HTB9-conditioned medium (HTB9-CM). The lower hatched area is the 95% confidence interval for blasts exposed to 2% HTB9-CM, the amount calculated as present in the media of blast cells 7 days after culture, in which the cultures were initiated with 10% HTB9-CM. Colony number stimulated by the addition of conditioned media is shown on the vertical axis. The percentage and source of conditioned media under test is shown along the horizontal axis. Triangles (left panel) show the number of colonies stimulated by 2% or 10% HTB9-CM incubated for 7 days in the absence of cells. The activity was stable under these conditions. In the center and right-hand panels, open symbols represent results of supernatants from blast cells cultured in the absence of 5-aza (5-aza); solid symbols represent supernatants of cultures in the presence of the drug. The middle panel (square symbols) shows results using cells that required HTB9-CM for growth; 20% conditioned media from these cells were less effective than the control values for HTB9-CM incubated for 7 days. Addition of 10% HTB9-CM to the blast supernatant yielded an activity equivalent to 10% HTB9-CM alone. The right panel (circles) shows data from cultures of cells that grew well without the addition of HTB9-CM. Supernatants from these cultures, with or without 5-aza, failed to stimulate blast colony formation. However, addition of 10% HTB9-CM gave the anticipated activity. Thus, blast cells in suspension did not secrete growth factors, nor did they when inhibitors were present.

5-aza was added at the time of culture initiation. Cells that grew without added HTB9-CM did not secrete blast growth factors when cultured for 7 days either with or without 5-aza. Controls in which HTB9-CM was added to supernatants from blast cultures were effective stimulators, indicating that residual cytotoxic drug or other inhibitors were not demonstrated in the supernatants.

Repeated exposure to 5-aza. The effects of 5-aza on blast progenitor self-renewal made it reasonable to question whether one more exposure to the drug would prolong the time for which blast progenitors could be maintained in suspension. Figure 8 shows the results of an experiment in which control and 5-aza-treated blast progenitors were maintained in suspension culture for 56 days. As in previous experiments of this design, the cells were harvested at weekly intervals, counted, washed, and recultured at 10^6/mL. The data are presented as a cumulative growth curve, calculated on the basis of the changes occurring in each weekly culture period. One experimental group was exposed to 5-aza (0.5 x 10^-6 mol/L) during the first week only; a second group was treated a second time between days 21 and 28. As anticipated from the 5-aza survival curves for blasts in suspension, the single drug exposure led to a slight decrease in clonogenic cells after 7 days. Thereafter, the treated group contained more progenitors than did the control for 49 days; both then declined rapidly. The second drug treatment led to an immediate cessation of growth; the cultures could be maintained through only one subsequent passage. In this and three similar experiments, exposure to 5-aza did not prolong time in culture. Furthermore, the cytotoxic effects of a second drug treatment were much greater than at the first exposure.

DISCUSSION

The work described in this paper was undertaken to test and extend our previous observation that blast cells were more sensitive to ara-C when exposed in suspension than in methylcellulose culture. We proposed that this difference might be explained by findings indicating that ara-C was a more effective inhibitor of blast cell renewal than of terminal divisions. Thus considered, increased sensitivity in suspension reflected the predominant role of renewal in this assay whereas the terminal divisions occurring in methylcellulose were less affected by the drug. This hypothesis led to the prediction that a cytotoxic agent that increased the probability of renewal in surviving stem cells would be less toxic in suspension than in methylcellulose. 5-Azacytidine has been shown to increase PE2 and was chosen for the test. Figure 1 shows that three classes of comparisons may be found when survival curves are measured for the same cells in suspension and methylcellulose. For adriamycin, an agent that is equally toxic for renewal and terminal divisions, the survival curves in suspension and methylcellulose were indistinguishable. As noted previously, ara-C was more sensitive in suspension than in methylcellulose. As predicted by the hypothesis, the findings with 5-aza were the reverse of those with ara-C: that is, the 5-aza D_50 was greater for blasts exposed in suspension than for the same populations cultured with the drug in methylcellulose.

The validity of survival curves obtained using either the clonogenic assay in methylcellulose or growth in suspension is dependent on the linear relationship between input cells and the manifestations of growth after 7 days; however, when results obtained with the two assays are compared, the cellular events leading to the endpoints for each must be
considered. Colony formation in methylcellulose requires that a progenitor divide often enough to have at least 20 progeny. The natures of the divisions, renewing or terminal, do not enter into the assessment; neither is there added weight for large colonies. A kinetic component of the methylcellulose assay might emerge if the times of incubation were prolonged; however, this is not practical experimentally, since colonies usually disintegrate later. In contrast, the assessment of the suspension assay reflects the number of renewing divisions occurring in 7 days; consequently, the measurement is very kinetics-dependent. The significance is apparent from the kinetic data presented in Fig 5, in which it is evident that the number of clonogenic cells recovered 7 days after exposure to 5-aza depends on both the inhibitory effects of drug during the 3 days it remains active and the subsequent recovery. A comparable recovery in colony formation in methylcellulose is unlikely because, as noted earlier, the colonies disintegrate after 7 to 8 days in culture and the number of clonogenic cells that can be recovered from the dishes also decreases markedly. These findings are compatible with the postulate that conditions in methylcellulose are less favorable for growth than are conditions in suspension.

We chose to retain the 7-day interval for both assays as the basis for comparisons between them, even though this allows time for recovery in the suspension assay. The advantage is that regrowth of malignant stem cells after chemotherapy, a clinically important function, is retained. Studies in which the cells were exposed briefly to 5-aza in suspension and then either cultured in suspension or methylcellulose for 7 days also showed, however, that the progenitors were more resistant in suspension than in methylcellulose. Other experiments, in which blasts were exposed in suspension for 3 days and then plated in methylcellulose gave comparable results. Thus, it is improbable that the findings with 5-aza can be explained by the kinetics of recovery of drug-exposed cells.

The mechanisms underlying the generation of different survival curves in suspension and methylcellulose remain to be determined. We excluded drug-induced secretion of growth factors following treatment of cultured cells with either ara-C or 5-aza (Fig 7). Selection in suspension of a resistant clone caused by the kinetics of recovery of drug-exposed cells. The comparison of the effects of 5-aza and 6-aza may contribute to the explanation of the observed phenomenon (Fig 6). DNA hypomethylation is a known effect of the former but not the latter. In the clonogenic assay, 5-aza is more toxic than 6-aza; the reverse occurs when the suspension method is used. Thus, DNA hypomethylation may contribute to either the inhibitory effects of 5-aza in suspension or recovery from them. It should be noted that $D_{50}$ 6-aza values are greater in suspension than in methylcellulose, however, although the difference between the two culture conditions is significantly less than for 5-aza.

Despite its effect of self-renewal, 5-aza treatment did not facilitate the establishment of continuous lines. Instead, toxicity of the drug appeared to be cumulative, since a second exposure, 2 weeks after the first, with two intervening subcultures, led to a much greater decrease in growth than anticipated on the basis of the survival curves or response to a single exposure. The mechanism underlying these observations is unknown. It is striking, however, that blast cells, so aggressive in patients, cannot regularly be maintained indefinitely in culture. It may be that further improvements in the culture techniques are required to achieve long-term growth.

Finally, the hypothesis that differences in survival curves measured in suspension as compared with methylcellulose may reflect specific drug effects on self-renewal leads to the prediction that a correlation may be found between $D_{50}$ values in suspension and clinical outcome. For ara-C, preliminary evidence is available in support of the prediction. Unfortunately, the blasts used for the 5-aza experiments were obtained from patients at initial presentation and at relapse. This heterogeneity in the patient population is sufficient to prevent valid statistical analysis. More survival curves on blasts from newly diagnosed patients are now being collected.

REFERENCES


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