A Cell Line Secreting Stimulating Factors for CFU-GEMM Culture

By C.D. Myers, F.E. Katz, G. Joshi, and J.L. Millar

The multipotent hemopoietic stem cell has fastidious growth requirements in vitro. Traditionally, phytohemagglutinin-stimulated leukocyte conditioned medium has been used to supply the undefined growth factors required for culture of the human multipotent hemopoietic progenitor. We describe the use of medium conditioned by the bladder carcinoma cell line, 5637, to replace PHA-LCM in CFU-GEMM cultures and show that the properties of this conditioned medium closely mimic those of PHA-LCM in two separate CFU-GEMM culture systems.

THE GROWTH OF multipotent hemopoietic colonies (CFU-GEMM) in vitro, first described by Fauser and Messner, depends on the presence of a number of factors, many of which are not yet identified. The usual source of these growth factors is phytohemagglutinin-stimulated leukocyte conditioned medium (PHA-LCM), produced by a 1-week culture of peripheral blood mononuclear cells stimulated by phytohemagglutinin. Conditioned medium prepared in such a way is subject to variation, which necessitates the production and testing of a number of batches in order to obtain a suitable colony-stimulating activity (CSA) source.

The bladder carcinoma cell line, 5637, produces colony-stimulating factor(s) for myeloid colony (CFU-GM) growth from both normal and malignant (CML) bone marrow. The 5637 conditioned medium has also been found to be active in stimulating the growth of mixed granulocytic colonies from murine bone marrow (D. Patinkin, E.R. Stanley, and T.R. Bradley, unpublished observations). We report that 5637 conditioned medium is also effective in supplying the stimulatory factors required for the culture of CFU-GEMM from human marrow and can, therefore, be used to replace PHA-LCM in these cultures.

MATERIALS AND METHODS

Cell Line 5637

The bladder carcinoma cell line 5637, originated by Dr G. Cannon, was kindly provided by Dr J. Fogh, Sloan Kettering Institute for Cancer Research, Walker Laboratory, Rye, NY, and was passaged routinely every 2 weeks in alpha-medium supplemented with 10% fetal calf serum. We describe the use of medium conditioned by the bladder carcinoma cell line, 5637, to replace PHA-LCM in CFU-GEMM cultures and show that the properties of this conditioned medium closely mimic those of PHA-LCM in two separate CFU-GEMM culture systems.

CFU-GEMM Assays

CFU-GEMM cultures were performed using a modification of the method described by Fauser and Messner. Bone marrow was obtained either from consenting adult volunteers by aspiration from the posterior iliac crest or from patients undergoing hip replacement surgery. Mononuclear cells were collected by centrifugation over Lymphoprep (Nyegaard and Co, Oslo, Norway) or 60% Percoll (Pharmacia, Uppsala, Sweden). Cultures were performed independently by two investigators, designated series 1 and series 2, each using separate reagents and different sources of PHA-LCM for comparison with the 5637 conditioned medium. In series 2, all marrow samples used were depleted of adherent cells by incubation at low cell concentration (<1 x 10^6/mL), in the presence of 20% FCS in tissue culture grade Petri dishes, either for four hours or overnight. The nonadherent cells were then removed by gentle washing. This procedure was also applied to some of the marrow samples used in series 1. Generally, 10^7 washed bone marrow mononuclear cells in Iscove's Medium (IMDM, Gibco, Paisley, Scotland) were cultured in the presence of 10% conditioned medium, 10% fetal calf serum (Sera Lab, Crawley Down, Sussex, or Gibco Bio-Cult, Glasgow, Scotland), methylcellulose at a final concentration of 0.8%, 5 x 10^-3 mol/L 2-mercaptoethanol, and 20% human plasma in a final volume of 2.7 mL. After addition of the human plasma and rapid mixing, 0.9-mL aliquots were placed in 30-mm Petri dishes containing 1 U of erythropoietin (either Connaught, Step III, or from the Terry Fox Laboratories, British Columbia Cancer Research Centre, Vancouver, Canada). The plates were incubated at 37°C in a humidified atmosphere containing 5% CO_2, and all colony types were scored using an Olympus inverted microscope after 13–15 days of culture.

RESULTS

A number of batches of 5637 conditioned medium was tested, two of them extensively. Table I shows that both of the main batches tested contained all the stimulatory factors necessary for CFU-GEMM growth. However, the batch based on TC-199 consistently performed better, and, in one series of tests, produced better growth of CFU-GEMM than the control PHA-LCM. The 5637 conditioned medium...
produced good numbers of CFU-GM, but there were usually decreased numbers of BFU-E and CFU-Meg. Other batches of 5637 conditioned medium examined showed batch variation in stimulatory activity, although the ratio of colony types obtained appeared to be consistent (Table 2). Two further batches of 5637 conditioned medium were tested for their ability to stimulate erythroid differentiation in the absence of erythropoietin. Both of these preparations were active using the normal culture conditions, however no erythroid or CFU-GEMM colonies were observed in cultures lacking exogenous erythropoietin. Two further sources of conditioned medium have been examined. GCT conditioned medium (Gibco-Biocult) stimulated GM colonies well but was a very poor stimulator of other colony types (C. Myers, unpublished observations). The EJ bladder carcinoma cell line5 was unable to stimulate the growth of GM colonies and was therefore not examined further (J. Millar, unpublished observations).

In another series of experiments, the linearity of the assay using 5637 conditioned medium was compared to that using traditional PHA-LCM. The results show that the assay is linear between 0.5 and $2.0 \times 10^5$ cells/mL plated for all colony types (Fig 1). The discrepancy between the megakaryocyte colony numbers is due to the different human plasma sources used in the two series; higher megakaryocyte counts were routinely seen in series 2. The stimulatory effect of the 5637 supernatant was shown to diminish when used at less than 5% and had an optimal concentration between 5% and 10% (Fig 2). This is similar to the titration of PHA-LCM (unpublished observation). However, higher concentrations of 5637 supernatant in the culture mixture were toxic to colony growth (Fig 2). The high residual level of CFU-GM in the absence of any exogenous stimulator is due to the human plasma used in the assay. The stimulating effect was maintained, though not significantly improved, when the 5637 medium was concentrated tenfold by ultrafiltration over an Amicon YM10 filter. The potency of the tenfold concentrate was maintained after freezing (data not shown).

**Table 2. Colony Growth Using 10% 5637 Conditioned Media**

<table>
<thead>
<tr>
<th>Number of Tests</th>
<th>Colonies (% of Control)</th>
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<tbody>
<tr>
<td></td>
<td>CFU-GM</td>
</tr>
<tr>
<td>Series 1</td>
<td></td>
</tr>
<tr>
<td>5637 TC199</td>
<td>1</td>
</tr>
<tr>
<td>5637 Alpha</td>
<td>1</td>
</tr>
<tr>
<td>5637 Hams</td>
<td>1</td>
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<tr>
<td>5637 Iscove</td>
<td>1</td>
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</tbody>
</table>

Percentage results for the number of colonies grown using separate batches of 5637 conditioned medium (not shown in Table 1) compared to the normal PHA-LCM used by two different investigators. Each set of results shows the mean for the number of tests listed. The test and control results were the mean of duplicate plates.

**DISCUSSION**

PHA-LCM, the traditional conditioned medium used in mixed colony cultures, has the problems of being both tedious and expensive to prepare and showing considerable batch variation. Batches are also usually of limited volume, making the biochemical analysis of the growth factors difficult.

We have shown that the bladder carcinoma cell line 5637 will secrete conditioning factors into its growth medium that can replace PHA-LCM in mixed colony cultures. Although there are some obvious differences in the potency of this preparation, particularly in its ability to stimulate the growth of BFU-E and CFU-Meg, these differences are likely to be due to the balance of the factors rather than any specific deficien-
The plating efficiency of colonies in the CFU-GEMM assay when stimulated by (A) 5637 conditioned medium, (B) PHA-LCM. Panel A is mean (± standard deviation) from six experiments, three in each of the two series. Panel B is mean (± standard deviation) for three experiments in series 1.

However, it is clear that 5637 conditioned medium, in common with PHA-LCM, does not contain erythropoietin, and this must be added from an exogenous source. The 5637 conditioned medium appears to provide a better stimulatory source for CFU-GEMM growth than PHA-LCM. We think that this is the most important feature of this conditioned medium, as each of the other colony types can be cultured in separate assay systems.

The range of linearity of our assay systems with respect to number of cells plated does not agree with that of Ash et al, who reported linearity of CFU-GEMM up to $10^6$ cells/plate. However, this does not appear to be due to the 5637 conditioned medium, as similar results were obtained using two separate batches of PHA-LCM. The maintenance of high levels of CFU-GM in the absence of exogenous growth factors suggests that GM–CSF is endogenous in these cultures. This is probably present in the human plasma added, as the other likely sources, monocytes and macrophages, have been largely removed by adherence in many of these experiments.

Cell lines secreting various growth factors have been described previously. Mouse L cells produce colony-stimulating activity and the murine WEHI-3 line produces many factors, including burst-promoting activity (BPA). Human lines producing CSA and erythroid potentiating factors are also known, but to date, only one line (Mo) has been shown to produce all the growth factors necessary to stimulate human CFU-GEMM growth. This and other similar HTLV-II–transformed, factor-secreting lines are available, but have the disadvantage of secreting active HTLV-II virions (David Golde, personal communication).

The advantages of a cell line secreting these factors is the convenience of producing large amounts of conditioned medium quickly and easily and with limited batch variation. This should improve both the
long-term reproducibility of the assay and also facilitate the biochemical analysis of early-acting growth factors, as previously described.\textsuperscript{15,16} Indeed, the capacity to produce growth factors in serum-free medium fulfills one of Stanley and Guilbert’s\textsuperscript{16} major criteria for this type of analysis.

ACKNOWLEDGMENT

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REFERENCES

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