Unicellular or Multicellular Origin of Human Granulocyte-Macrophage Colonies In Vitro

By Jack W. Singer, Philip J. Fialkow, Lois W. Dow, Connie Ernst, and Laura Steinmann

The assumption that human granulocyte-macrophage colonies have a unicellular origin and thus are true clones has been directly tested. Cells from seven females heterozygous for the common glucose-6-phosphate dehydrogenase (G-6-PD) gene (GdB) and the variant GdA were cultured in semisolid medium for granulocyte-macrophage colony growth and the enzyme type of individual colonies was determined. When the colony density was less than 20/dish, more than 95% of colonies had either type A or type B G-6-PD, but not both. At colony densities >30/dish, between 15% and 75% of colonies had both enzyme types and therefore arose from more than one cell. These results are consistent with a unicellular origin for the colonies only when they are cultured at low densities. With increasing colony density, there was a greater frequency of colonies with both type A and type B activity, suggesting that accurate enumeration of committed stem cells can only be performed at low colony concentrations.

The development of techniques for culturing human hematopoietic cells in semisolid media has been used as a quantitative tool for enumerating committed granulocyte-macrophage progenitors (CFU-C) and factors that control their proliferation. Implicit in many of these studies is the assumption that each colony is a true clone, i.e., that it is derived from a single progenitor CFU-C and, therefore, that the number of colonies that grow is an accurate reflection of the number of progenitors plated.

This question has been studied by using a naturally occurring system of cellular mosaicism, the X-linked glucose-6-phosphate dehydrogenase (G-6-PD) isoenzyme locus as a marker. Due to random inactivation of one of the two X chromosomes in each somatic cell that occurs early in embryogenesis, women heterozygous for the usual G-6-PD gene (GdB) and a variant, such as GdA, have two populations of cells, one synthesizing type B and the other type A G-6-PD. All the cells in a colony with a unicellular origin should contain either type B or A enzyme, depending on whether the single progenitor cell had GdB or GdA on the active X chromosome. Colonies with a multicellular origin should contain variable ratios of both G-6-PD types.

MATERIALS AND METHODS

Four of the G-6-PD heterozygotes studied are hematologically normal (patients 1–4). Patient 5 had acute nonlymphocytic leukemia in remission, and patients 6 and 7 had acute lymphocytic leukemia in remission.

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Peripheral blood was collected in preservative-free heparin (10 U/ml) and the buffy coat cells were separated. The cells were lysed by freezing and thawing in Tris-HCl buffer (pH 8.0) containing NADP, 2-mercaptoethanol and EDTA. The cell lysates were subjected to starch gel electrophoresis and the relative activity of the isoenzyme bands was estimated visually. Cells for culture were separated on discontinuous density gradients (Teva, Jerusalem). The interphase cells were washed twice in Hank's balanced salt solution (Microbiological Associates) and the viable cells were enumerated by trypan-blue dye exclusion.

For colony growth, between 0.5 and 10 × 10⁶ cells/ml were plated in a mixture consisting of 0.8% methyl cellulose (MCB) or 0.3% agar (Bactoagar; Difco, Detroit, Mich.) in alpha medium (Flow Labs, Rockville, Md.) containing 20% fetal calf serum (Reheis) and a plateau dose of phytohemagglutinin (PHA) conditioned medium. One milliliter aliquots of this cell mixture were plated in 10 × 35 mm plastic Petri dishes (Lux) and incubated for 14 days at 37°C in a high humidity, 5% CO₂-95% air, tissue culture incubator.

The colonies in each plate were counted with an inverted microscope at 40× and then individually electrophoresed to determine the G-6-PD type by previously published methods. Colonies were plucked with a fine capillary pipette with a dissecting microscope. Care was taken not to pluck colonies for electrophoresis that were closely adjacent to other colonies. A sample of supporting media without a colony was electrophoresed on each run to determine the amount of background activity in the dishes. In each experiment, random individual colonies similar in appearance to those electrophoresed were plucked and shown to stain positively for peroxidase and not to form rosettes with sheep erythrocytes. Two experimental colonies and one control, type B colony grown from a normal GdB male, were placed on each cellulose acetate strip. Lysis of the colony cells was achieved by freezing with dry ice. The strips were immediately subjected to electrophoresis and then stained for G-6-PD activity. In preliminary experiments, it was determined that the G-6-PD type of clusters as small as 20 cells could be determined.

RESULTS

The ratio of A:B G-6-PD activity in red cells, buffy coat cells, or skin fibroblasts from the heterozygotes are shown in Table 1, as well as the G-6-PD isoenzyme types of individual granulocytic colonies and the number of colonies harvested in each dish. When the number of colonies per dish was low, almost all of the colonies showed only one enzyme type, but as colony concentrations increased, the prevalence of colonies with a double isoenzyme type rose (p < 0.001; r = 0.92; Spearman’s rank order correlation coefficient). The morphology of each colony was

Table 1.

<table>
<thead>
<tr>
<th>Patient* No.</th>
<th>A:B Ratio of G-6-PD</th>
<th>Buffy Coat Cells</th>
<th>Skin Sample</th>
<th>G-6-PD Type of Individual Granulocyte Colonies</th>
</tr>
</thead>
<tbody>
<tr>
<td>RBC</td>
<td></td>
<td></td>
<td>Colonies/Dish</td>
<td>A</td>
</tr>
<tr>
<td>1</td>
<td>1:1</td>
<td>1:1</td>
<td>PB</td>
<td>5</td>
</tr>
<tr>
<td>2</td>
<td>2:3</td>
<td>1:1</td>
<td>PB</td>
<td>12</td>
</tr>
<tr>
<td>3</td>
<td>5:1</td>
<td>5:1</td>
<td>PB</td>
<td>2</td>
</tr>
<tr>
<td>4‡</td>
<td>2:3</td>
<td>1:1</td>
<td>PB</td>
<td>9</td>
</tr>
<tr>
<td>5‡</td>
<td>2:1</td>
<td>3:1</td>
<td>PB</td>
<td>55</td>
</tr>
<tr>
<td>6‡</td>
<td>1:1</td>
<td>1:1</td>
<td>Marrow</td>
<td>110</td>
</tr>
<tr>
<td>7‡</td>
<td>1:1</td>
<td>1:1</td>
<td>Marrow</td>
<td>(See Fig. 1)</td>
</tr>
</tbody>
</table>

*t: Mean ± 1 standard deviation.
‡: Studies on this patient have been published previously.

A:B ratio of G-6-PD isoenzyme activity in cells from 7 G-6-PD heterozygotes treated directly. Patients 1–4 are hematologically normal. Patient 5 had acute nonlymphocytic leukemia in complete remission at the time of study. Patients 6 and 7 had acute lymphocytic leukemia in remission.
Fig. 1. The effect of increasing granulocytic colony density on the frequency of A/B colonies grown from peripheral blood mononuclear cells from patient 4. The solid line indicates the observed number of colonies/dish at each plating density, and the hatched line indicates the frequency of colonies with both A and B type G-6-PD activity.

Figure 1 shows a dose–response curve with cells from patient 4 cultured in methylcellulose and the frequency of colonies that typed as A/B at each point. No colony had a double-enzyme phenotype at densities of <12/dish. However, at 29/dish, 4 of 23 (17%) typed as A/B. Figure 2 shows a similar set of curves derived from marrow cells from patient 7 cultured in agar. At colony densities <25/dish, all colonies typed as either A or B, whereas when there were 60 or more colonies per dish, colonies with more than a single cell origin were found.

The visual estimation of the amount of enzyme per colony correlated not with cell number but with an estimation of the macrophage content of colonies. Mixed granulocyte-macrophage colonies containing as few as 20 macrophages often contained more activity than compact colonies containing several hundred granulocytes. All colonies analyzed in the cultures grown from the white male controls typed as B.

DISCUSSION

Although it is assumed on the basis of indirect evidence that each human granulocyte colony grown in vitro arises from a single cell, formal proof has not previously been reported. The best evidence supporting the hypothesis of a unicellular origin for granulocyte-macrophage colonies was reported by Moore et al., who micromanipulated single cells from monkey marrow and found that they described at the time of plucking for electrophoresis, and no differences were noted between colonies giving rise to both isoenzyme types and those with a single isoenzyme type. Even at high cell densities, electrophoresis of supporting material without a colony demonstrated no measurable G-6-PD activity.

Fig. 2. The effect of increasing granulocytic colony density on the frequency of A/B colonies grown from marrow from patient 7. The solid line indicates the observed number of colonies/dish at each plating density, and the hatched line indicates the frequency of colonies with both A and B type G-6-PD activity.
formed granulocyte-macrophage colonies. In earlier studies, Bradley and Metcalf, in a murine system, demonstrated a linear regression line for the numbers of murine colonies versus the numbers of cells plated and suggested that each colony arose from an individual cell.

To formally test the hypothesis of the unicellular origin of human granulocyte-macrophage colonies, we have studied the isoenzyme type of individual colonies grown from G-6-PD heterozygotes. G-6-PD mosaicism has been used previously to determine the number of cells from which human neoplasms arise, to show the clonal origin of day 8–13 human erythroid colonies and to study aspects of cell regulation in polycythemia vera and chronic myelogenous leukemia.

The data indicate that when plated at sufficiently low density, granulocytic colonies from subjects heterozygous for G-6-PD contain either type A or type B G-6-PD and therefore arise from single progenitors. If granulocyte colonies arose from two cells, 50% of the colonies should have had both isoenzyme types on electrophoresis. An even greater frequency of mixed A and B type colonies would be expected if they arose from three or more cells. The present electrophoretic results at low colony densities are clearly different from these predictions and are most consistent with origin of granulocyte/macrophage colonies from a single CFU-C.

The presence of increasing numbers of colonies with both isoenzyme types as colony density increases is consistent with the assumption that at least some of these colonies arose from two or more spatially closely related progenitors. Since A/B colonies arise from at least two progenitors, the actual frequency of colonies arising from more than a single cell is more than double the incidence of A/B colonies from balanced heterozygotes. Even if all colonies had a two-cell origin, 50% would still have a single enzyme type. Thus, in patient 4, at 29 colonies/plate, 17% were A/B; the frequency of colonies with a multicellular origin was at least 34%.

If the relationship between the number of CFU-C plated and the number of colonies produced is a simple one, the G-6-PD data predict that it should be linear only at colony densities up to approximately 30/dish. At higher densities, due to the increasing frequency of colonies with a multicellular origin, a dose–response curve with a continuously decreasing slope would be expected. However, in contrast to this prediction, the dose–response relationships are linear up to 160 colonies/dish (Fig. 2). Therefore, the relationship between number of CFU-C plated and number of colonies that grow out is not simple at higher cell densities. Possible explanations for the greater than expected number of colonies include cell–cell interaction resulting either in increased proliferative potential of cluster-forming cells so that they produce colonies, or recruitment of additional cells into the CFU-C compartment. Insofar as the putative increased growth resulting from direct cell–cell interactions is cell-density dependent, the dose–response relationship should be nonlinear with a continuously increasing slope. However, this response was not observed, probably due to the increasing number of colonies with a multicellular origin. Therefore, the observed linear cell dose–response curve may be complex and the result of the summation of two opposite nonlinear phenomena.

Two conclusions drawn from these data are: (1) The majority of granulocyte-macrophage colonies can be assumed confidently to have a unicellular origin only if plated at a cell density low enough to yield 30 or fewer colonies per dish; (2)
accurate enumeration of CFU-C is best performed only when cells are plated at low density. The multicellular origin of the colonies causes significant underestimation of the number of committed stem cells at colony concentrations as low as 30/dish, and the underestimation increases substantially as the colony density increases. In cultures with more than 100 colonies/dish, few if any colonies can be assumed to have a unicellular origin. Therefore, for accurate enumeration of CFU-C, a cell dose-response curve should be performed, and the absolute CFU-C concentration calculated from those dishes with fewer than 30 colonies. Only then can it be assumed that colonies arise from single progenitors. However, even under these circumstances because of the lack of stimulatory cell–cell interactions, all potential CFU-C may not form colonies.

Typing colonies from G-6-PD heterozygotes also should be helpful in definitively establishing whether or not other types of in vitro colonies such as the “mixed cell”\textsuperscript{2,13,14} and T-cell colonies\textsuperscript{3,4} arise from single progenitors. The present study demonstrates some of the pitfalls of assuming a unicellular origin for colonies, even when they are at relatively low densities and appear to have a linear cell-dose to colony-number relationship.

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**REFERENCES**
