The Effect of Monocytes in the Peripheral Blood CFU-C Assay System

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The effect of cellular interactions in the in vitro assay of myeloid progenitor cells in peripheral blood (PB CFU-C) was investigated. Ficoll-Paque-separated peripheral blood mononuclear cells (PB MNC) from 7 healthy subjects were cultured at cell concentrations from 10 to 0.625 × 10^6 MNC/plate in doubling dilutions. The number of colonies per 10^6 lymphocytes plated (corrected colony count, CC) was significantly higher when 2.5 × 10^6 or less PB MNC were cultured than when 5 or 10 × 10^6 cells were cultured. This decrease in CC when large numbers of cells were cultured was not present when the nonadherent cells only were cultured. The inhibition was reproduced when adherent cells were added back to the nonadherent cells. The inhibition appeared to be proportional to the number of monocytes present. A model depicting the role of monocytes in the PB CFU-C assay system is presented. The increased understanding of cellular interaction represents an important step towards the standardization of the PB CFU-C assay.

It is probable that the difference in cell populations sampled is an important cause of the variability in the PB CFU-C assay. In the PB CFU-C assay, the cells cultured are peripheral blood mononuclear cells, which consist mainly of lymphocytes and monocytes (60%-80% and 20%-40%, respectively). CFU-C are less than 0.1% of the cells present. In contrast, the cells cultured in the BM system consist of maturing myeloid and erythroid cells, with lymphocytes and monocytes constituting less than 20% of all cells. CFU-C again constitute less than 0.1% of the cells. Furthermore, the cell concentration used in the PB assay is usually much higher (5-10 × 10^5/ml) than that in the BM assay (1-2 × 10^5/ml). As a result, the number of lymphocytes and monocytes in the PB CFU-C assay may be up to 100 times more than that in the BM assay.

Differential lymphocyte subsets may exert important influences on hemopoiesis in certain diseases, but their role in the in vitro assay system is less well defined. Monocytes secrete colony-stimulating factors (CSF) necessary for the growth of CFU-C, as well as prostaglandin E (PGE) and acidic isoferritins (AIF), which are inhibitory to the CFU-C. The net colony stimulating activity is therefore a balance between these antagonistic influences. Because of the larger number of monocytes present in the PB assay system compared with the BM system, any change in the net modulatory influence of the monocyte population may cause an apparent change in the level of CFU-C. Experiments were therefore performed to determine whether cellular interactions play an important role in the in vitro PB CFU-C assay.

MATERIALS AND METHODS

Preparation of Cell Fractions for Culture

Blood was collected by venipuncture from 7 healthy volunteers between 8:30 a.m. and 9:30 a.m. Three of the volunteers were studied twice. The blood was diluted in an equal volume of Dulbecco’s phosphate-buffered solution (Commonwealth Serum Laboratories, Melbourne, Australia) and was then layered onto Ficoll-Paque
(specific gravity 1.077, Pharmacia, Uppsala, Sweden) and centrifuged at 400 g for 30 min. The mononuclear cells at the interface (fraction A) were collected and washed 3 times with RPMI 1640 cell culture medium (Flow Laboratories, Rockville, MD).

The fraction A mononuclear cells were separated by adherence to plastic. Washed cells were added to RPMI 1640 supplemented with 15% fetal calf serum (FCS, Commonwealth Serum Laboratories, Australia) in 90-mm plastic Petri dishes (Disposable Products, Australia) to give a cell concentration of 1.4–1.7 x 10^6 cells/ml in a volume of 10 ml and were incubated at 37°C in 5% CO₂. After 1 hr, the supernatant containing the nonadherent cells was transferred to another Petri dish, and the adherence procedure repeated. The nonadherent cells were then recovered by centrifugation of the supernatant (fraction B).

Fresh medium was added to the cells that adhered to the bottom of the plastic dishes, and incubation was continued for 1 hr more. The supernatant was discarded, and the adherent cells were recovered by scraping (fraction C).

Calculated volumes of each cell fraction were placed in 35-mm Petri dishes (Kayline, South Australia). The appropriate volumes of alpha-modified Eagle’s minimum essential medium (Flow Laboratories, Australia) with 15% FCS in 0.3% agar (Bacto-Agar, Difco Laboratories, Australia) were added so that cells were plated at 0.625, 1.25, 2.5, 5, and 10 x 10^3/plate. The final volume was 1 ml/plate. Quadruplicate plates were set up for each cell concentration. There were usually insufficient cells in fraction C, so that only the lower concentrations could be cultured.

The percentages of granulocytes, lymphocytes, and monocytes were determined on cytocentrifuge preparations stained by (1) the Jenner-Giemsa method, and (2) the combined specific and nonspecific esterase method. At least 400 cells were counted in each of these preparations.

Preliminary experiments have been carried out to compare the composition of the lymphocyte populations before and after the adherence procedure. The percentage of T lymphocytes was determined by the sheep red cell rosette method, and the percentage of B lymphocytes was determined by a direct immunofluorescence method using anti-human immunoglobulin sera. No significant difference was detected.

**Mixing Experiments (Fraction D)**

A differential count was first performed on fraction A to determine the proportions of lymphocytes and monocytes. Fraction B and fraction C were then mixed together in the following manner.

**Sets D1-D5.** These plates contained the same number of fraction B cells as in set D1, with an increasing number of fraction C cells so that the number of monocytes in each plate corresponded to the number of lymphocytes present in fraction A, 10 x 10^3 cells/plate.

**Sets D2-D5.** These plates contained the same number of fraction B cells as in set D1, with an increasing number of fraction C cells so that the number of monocytes in each of the D2, D3, D4, and D5 plates would correspond, respectively, to 12.5%, 25%, 50%, and 100% of the number of monocytes in each of the fraction A, 10 x 10^3 cells/plate. Set D5 therefore contained the same number of lymphocytes and monocytes as the fraction A 10 x 10^3 set. Quadruplicate cultures were set up for each set.

**CFU-C Culture**

Colony-stimulating activity was provided by adding 0.1 ml of human placental conditioned medium (HPCM) to each plate. This amount of HPCM has been shown to provide the optimal amount of exogenous CSF in the assay system in our laboratory. Two control plates with no added HPCM were set up for each experimental point. The cultures were incubated in a closed humidified environment, with an atmosphere of 7.5% CO₂, 7.5% O₂, and 85% N₂. Aggregates of more than 40 cells at day 14 were scored as colonies.

CFU-C levels are often expressed in colonies per 10⁶ mononuclear cells plated. However, as the cells are further fractionated, the ratio of CFU-C to total number of cells becomes different in the various cell fractions. As the PB CFU-C is a nonadherent mononuclear cell morphologically similar to a lymphocyte, it is reasonable to express CFU-C levels in colonies/10⁶ lymphocytes, because the CFU-C:lymphocyte ratio should be much less affected by the adherence procedure than the CFU-C:total cells ratio. The results in this report were therefore expressed as the number of CFU-C/10⁶ lymphocytes.

The results were calculated as follows: CC = (N x 10/P) x (100/LC), where CC is the number of CFU-C/10⁶ lymphocytes (corrected colony count), N is the number of colonies/plate, P is the number of cells/plate x 10⁶, and LC is the percentage of lymphocytes in cell fraction.

This study has been approved by the Research Review Committee, Royal Adelaide Hospital.

**RESULTS**

**CFU-C Assay Results Using Peripheral Blood Mononuclear Cells (Fraction A)**

This fraction consisted of 74% ± 9% lymphocytes and 21% ± 8% monocytes (mean ± 1 SD of 10 experiments). The growth pattern of the CFU-C in this fraction is shown in Fig. 1. The number of CFU-C/10⁶ lymphocytes plated (corrected CFU-C count, CC) of this MNC fraction was much higher when smaller numbers of cells were plated than when the usual 5 or 10 x 10⁶ cells were plated. A two-way analysis of variance showed significant differences in the CC when different numbers of cells per plate were cultured (p < 0.001). In all but 4 of the sets, the CC reached a maximum value when either 1.25 or 2.5 x 10⁵ MNC were plated. The CC at these 2 cell concentrations were significantly higher than the levels at 5 or 10 x 10⁶ MNC/plate (CC at 2.5 x 10⁵ MNC/plate compared with CC at 5 x 10⁵ MNC/plate, p < 0.005, paired t test). In 5 of the experiments, there was also a decrease in CC when 0.625 x 10⁶ PB MNC/plate were cultured. At this cell concentration, there were less than 0.2 x 10⁵ monocytes/plate. For the 3 subjects studied twice, the same pattern was observed on both occasions. The control plates with no added HPCM showed only a small number of colonies or none at all.

**CFU-C Assay Results Using Nonadherent Cells (Fraction B)**

This fraction consisted of 95% ± 3% lymphocytes (mean ± 1 SD of 9 experiments). The results of 9 sets of assays are shown in Fig. 2. The CFU-C growth pattern was entirely different in this nonadherent cell fraction when compared with that of the MNC fraction. A two-way analysis of variance showed significant differences between the CC when different numbers of nonadherent cells were plated (p < 0.002). In
all experiments, the CC was significantly higher at \( 5 \times 10^5 \) or \( 10 \times 10^5 \) cell/plate than at the lower cell numbers (\( 5 \times 10^4 \) levels compared with \( 2.5 \times 10^5 \) levels, \( p < 0.005 \), paired t test). The removal of monocytes from the original mononuclear cell preparation has resulted in higher levels of CFU-C growth at high plating numbers. For the 2 subjects studied twice, the same pattern was observed on both occasions. The control plates with no added HPCM showed only a small number of colonies or none at all.

**CFU-C Assay Results Using Adherent Cells (Fraction C)**

This fraction consisted of 79% ± 7% monocytes (mean ± 1 SD of 5 experiments). No assays could be performed at high cell numbers per plate because of lack of sufficient cells in this fraction. Culture at low cell numbers per plate yielded usually very low numbers of colonies per plate. There were less than 4 colonies in 70% of the plates, and no colonies could be detected in 30% of the plates. The culture results were therefore considered unsuitable for analysis with regard to the effect of plating numbers.

**CFU-C Assay Results in the Mixing Experiments (Fraction D)**

The results of the 5 sets of mixing experiments are shown in Fig. 3. Each set of results represents the effect of adding an increasing number of adherent cells to a constant number of nonadherent cells. The level of CFU-C decreased significantly as more adherent cells were added; the CC at D1 (no adherent cells added) compared with CC at D5 (highest number of adherent cells added) showed a highly significant difference, \( p < 0.0025 \), using paired t test. This therefore virtually reproduced the pattern when MNC were cultured.

**Summary**

The mean CC at each of the five cell concentrations of all the experiments was calculated. A mean value of the number of monocytes/plate was also calculated for each of the five cell concentrations. The means and standard errors of CC at the five cell concentrations were then plotted against the mean number of monocytes/plate at those points. This was done for CFU-C assay results on fractions A, B, and D. The three graphs are shown in Fig. 4. From this figure, 0.15–0.4 \( \times 10^5 \) monocytes/plate appears to be optimal for CFU-C growth in this in vitro assay system.
DISCUSSION

The significant decrease in CC when large numbers of peripheral blood mononuclear cells were plated has not been reported before. There is one report claiming that the number of colonies grown per plate increased as the number of PB MNC plated was increased,\textsuperscript{15} which is contrary to our findings. Their assay system, however, was different because the presence of added CSA did not increase CFU-C growth. There is another report showing an increase in the number of colonies grown per plate as the number of whole-blood leukocytes plated was increased,\textsuperscript{7} but the same report also showed an unusual finding in that the number of colonies grown per 10\textsuperscript{6} whole-blood leukocytes was similar to the number of colonies grown per 10\textsuperscript{6} PB MNC. The discrepancy between these findings may at least in part be due to the effect of monocytes on the PB CFU-C assay, as discussed below.

The pattern of decreased CC at high plating numbers of PB MNC suggests the presence of an inhibitory...
influence related to the number of cells plated. The inhibition appears to be due to the large number of monocytes present because their removal resulted in a marked increase in CC. Furthermore, this pattern of monocyte-dependent inhibition was reproduced by adding the monocyte-rich fraction to the lymphocyte-rich fraction in the mixing experiments. Increasing inhibition of CFU-C growth was evident when increasing numbers of adherent cells were mixed with a constant number of nonadherent cells. The same pattern of inhibition was observed when higher concentrations of HPCM were used, so it is not due to excess utilization of CSA by monocytes. The same culture system has supported the growth of over 400 colonies/plate, so the decreased CC at higher plating numbers is not caused by nutritional deprivation. All the findings appear to suggest that monocytes present in a concentration of 0.4 \( \times 10^4/\)plate or more are inhibitory to CFU-C growth in the in vitro PB CFU-C assay system.

There are also indications that too few monocytes/plate may be suboptimal for CFU-C proliferation. A decrease in CC was observed when the number of monocytes/plate was less than 0.2 \( \times 10^5 \) in 5 of 10 experiments (Fig. 1). The CC in the nonadherent fraction also decreased when the number of monocytes/plate was less than 0.15 \( \times 10^5/\)plate (Fig. 2).

There is a distinct advantage in using CFU-C/lymphocyte rather than CFU-C/total cells to express the assay results. Assay results expressed in CFU-C/total cells showed an effect related to the number of cells plated and to the composition of the cells plated. The use of CFU-C/lymphocyte demonstrates more convincingly that the effect is related to the number of monocytes present in the culture.

The monocyte-macrophage system has been shown to have important regulatory influences on CFU-C growth. Monocytes are an important source of CSA, which is essential for the proliferation and maintenance of CFU-C. It has also been claimed that monocytes may be important in the “processing” of CSA. Until recently, it has been thought that monocytes play a definite, though small, stimulatory role in the CFU-C assay system. However, this conclusion is based on the BM system, where the number of monocytes is very small. When the effect of a larger number of monocytes on the marrow system was studied, it was found that monocytes secreted PGE, which inhibited CFU-C proliferation, and this PGE secretion was greatly enhanced by the presence of CSA. While such high numbers of monocytes are not usually present in the BM CFU-C assay, this regularly occurs in the PB CFU-C assay. AIF derived from monocytes has also been demonstrated to be inhibitory to CFU-C proliferation. Moreover, AIF production is also dependent on the presence of a critical number of monocytes. With this dual stimulatory/inhibitory role of monocytes in mind, a model can be constructed to explain the particular growth pattern of CFU-C (Fig. 5).

When small numbers of PB MNC are plated (Fig. 5, region I), the stimulatory effect of exogenous CSA predominates (line 1). Optimal CFU-C growth, however, is not achieved. This could be due to an inadequate cell–cell interaction or an insufficient number of monocytes for the processing of CSA.

As the number of cells per plate is increased, the number of monocytes plated is also increased, and optimal CFU-C growth is achieved (Fig. 5, region II). When the number of cells per plate is increased further, resulting in more than 0.4 \( \times 10^5 \) monocytes present per plate, there is a sudden decrease in CFU-C growth (region III); this effect may be due to humoral inhibitors secreted by monocytes like PGE or to cell–cell interaction, leading to release of humoral inhibitors and/or contact inhibition at high cell number/plate. The net colony-stimulatory activity in the culture system decreases, and the apparent level of CFU-C (or CC in practical terms) consequently decreases.

Most reported studies on PB CFU-C are based on assays performed on PB MNC without allowance for

![Fig. 5. A model depicting the proposed regulatory role of monocytes in PB CFU-C assay. Line 1 (-----) represents the exogenous colony-stimulating factor(s) added to the assay system in the form of HPCM. Line 2 (--.--.--.) represents the monocyte-derived colony-stimulating factor(s), the activity of which is proportional to the number of monocytes present. Line 3 (-----) represents the total colony-stimulating activity in the assay system. This is the sum of the exogenous and the endogenous colony-stimulating factors. Line 4 (-----) represents the inhibitory activity in the assay system, such as humoral inhibitors secreted by monocytes or due to cell–cell interaction. Line 5 (-----) represents the net colony-stimulating activity in the assay system as reflected by the observed CFU-C/10^5 lymphocyte level. In effect, line 5 is the difference between the total colony-stimulating activity (line 3) and the inhibitory activity (line 4).](image-url)
the effect of monocytes, and the plating number used varies widely. The present study showed that the measured level of PB CFU-C can be affected by the number of monocytes per plate, so results reported by different workers may not be comparable to one another.

The present study provides strong evidence that the monocyte-macrophage system may play an important regulatory role in granulopoiesis, and further advances in this direction may provide more insight into the control of hemopoiesis in vivo. This study also represents a significant step towards the establishment of a quantitative and reproducible assay of the PB CFU-C. Such an assay can then be used to elucidate the reliability and usefulness of the circulating CFU-C level as an index of hemopoietic activity.

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