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# Separation of Agar Colony Forming Cells from Rat Bone Marrow[1] (37843)

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Utilizing techniques introduced by Bradley and Metcalf (1), bone marrow cells have been shown to form colonies in agar after stimulation by a principle termed the colony stimulating factor (CSF). At a given concentration of CSF, there is a linear relation between the number of cells seeded and the number of colonies produced, and this relation has formed the basis of the assay for the *in vitro* agar colony forming cell (2).

The element responsible for *in vitro* myeloid colony formation (CFC) is thought to be derived from the hematopoietic pluripotent stem cell (CFU) and to be committed to myeloid differentiation. Varying degrees of success have been reported for the enrichment of agar colony forming cells using buoyant density or velocity sedimentation separation of mouse hematopoietic tissue (3–5). However, until relatively recently (6), no morphological characterization of the cells represented by these fractionation techniques and responsible for colony formation has been reported.

The present study has concerned the application of discontinuous equilibrium density gradient separation to rat bone marrow cells. Each cell fraction was placed in agar culture with and without CSF. Analysis of the different cell types present was performed in an attempt to associate morphologically a particular cell type with the formation of agar colonies.

*Materials and Methods.* Colony stimulating activity has been reported in the serum of 200–250 g male Long–Evans rats which had been injected with endotoxin (7). Samples of peripheral blood were collected from the tail vein at 0.5, 2, 4, and 6 hr following iv injection of 4 $\mu$g of *E. coli* endotoxin (Difco) in 0.2 ml saline and analyzed for total and differential white blood cell counts. At the same time, blood was collected aseptically from the dorsal aorta, centrifuged, and the serum collected after filtration through a 0.45-$\mu$m Millipore filter.

Bone marrow cells from 150-g male Long–Evans rats which had been previously exposed to 0.45 atm of air for 7 days and subsequently returned to normal atmospheric conditions for an additional 8 days were used in all experiments reported. Such marrow has been shown to contain very few nucleated red cells, thus eliminating a cell type not involved in myeloid colony formation. Bone marrow cells were removed from the tibias and femurs of rats by aspiration with Eagle's Minimum Essential Medium (MEM). A single cell suspension was obtained by gently pipetting the marrow up and down and straining through a 100-mesh stainless steel screen. A modification of the mouse agar culture technique of Metcalf (2) was used. Double-strength modified Eagle's Medium containing 10% heat-inactivated newborn calf serum, 10% heat-inactivated bovine serum, 20% trypticase soy broth, 75 $\mu$g/ml DEAE dextran

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TABLE I. Changes in Peripheral White Blood Cells/mm³ at Varying Times After Injection of 4 μg Endotoxin.

| Time after injection (hr) | Total white cell count (cells/mm³) | Granulocytes (cells/mm³) | Mononuclear cells (cells/mm³) |
|---|---|---|---|
| 0.0 | 13270 ± 948[a] | 2531 ± 310 | 10725 ± 766 |
| 0.5 | 10100 ± 1353[b] | 1934 ± 491 | 8166 ± 892 |
| 2.0 | 14560 ± 1882 | 9907 ± 1473[c] | 4603 ± 567[c] |
| 4.0 | 19930 ± 1349[c] | 14682 ± 930[d] | 4991 ± 1063[c] |
| 6.0 | 27850 ± 2784[c] | 22383 ± 2794[d] | 5467 ± 102[c] |

[a] Each figure is based on experiments with 5 rats/time point and expressed as the mean ± 1 SEM.

[b] $P < .10$ when compared to preinjection level.

[c] $P < .01$ when compared to preinjection level.

[d] $P < .001$ when compared to preinjection level.

(200,000–300,000 mol wt), 20 μg/ml L-asparagine, and 1 ml penicillin–streptomycin–mycostatin solution was added to an equal volume of 0.6% agar in deionized water. Three milliliters of this agar medium containing $10^5$ nucleated bone marrow cells/ml were pipetted into 60 × 15 mm petri dishes (Falcon) supplied with a suitable source of CSF. They were thoroughly mixed and the cultures allowed to gel at room temperature. Incubation proceeded at 37° for 7–10 days in a humidified atmosphere of 5% $CO_2$ in air. At this time, discrete colonies appeared and were counted under a low-power binocular microscope. Their numbers were compared to the number of cells cultured and to the amount of CSF added.

The direct effect of endotoxin on colony formation by rat bone marrow cells was evaluated in one experiment. For this study, varying amounts of endotoxin, after incubation with normal rat serum for 2 hr, were added to plates without CSF. The number of colonies formed was compared to the number of colonies obtained with normal rat serum without endotoxin addition.

For separation studies, rat bone marrow cells were resuspended in 17.0% bovine serum albumin (BSA) at a concentration of $10^8$ nucleated cells/ml and fractionated using a modified discontinuous albumin density gradient centrifugation technique of Dicke *et al.* (8). Stock solutions of bovine serum albumin, fraction V (Sigma) 35.0% wt per vol, were prepared in 0.155 *M* Tris buffer (pH 7.2) and stored at −20°. Different BSA concentrations were obtained by diluting the 35.0% BSA stock solution with 0.06 *M* phosphate buffer containing 0.154 *M* NaCl. One milliliter portions of each BSA solution (from heaviest to lightest) were layered carefully in a round-bottom siliconized centrifuge tube (16 × 0.8 cm).

The sequence of percentages employed were as follows: 35.0, 31.0, 29.0, 27.0, 26.5, 26.0, 25.5, 25.0, 23.0, and 17.0% BSA containing the cells in suspension. The tubes were centrifuged for 30 min at 1000*g* in an International Centrifuge equipped with a swing-out head. After centrifugation distinct cell layers were visible near the gradient interfaces and each layer was collected for culturing and for differential cell counts.

*Results. Effect of endotoxin injection on peripheral white blood cells and serum colony stimulating activity.* Changes in total WBC, granulocytes, and mononuclear cells/mm³ after an iv injection of 4 μg of endotoxin are given in Table I. During the initial 0.5 hr after injection, absolute counts of total WBC decreased insignificantly ($.05 < P < .10$). A significant increase occurred through the next 5.5 hr to about double the baseline (0 hr) value ($P < 0.01$). During this time, granulocytes, after a nonsignificant initial drop, increased significantly ($P < 0.001$) by 6 hr, accounting for the rise seen in total WBC. Mononuclear cells, however, exhibited a progressive decline which reached its nadir ($P < 0.01$) 2–6 hr after endotoxin treatment.

TABLE II. Changes in Serum Colony Stimulating Activity at Varying Times after Injection of 4 μg Endotoxin.

| Serum type | Amount used (ml) | Number of colonies/ $10^5$ cells |
|---|---|---|
| Normal rat serum | 0.6 | 8 ± 0.92[a] |
| 0.5-hr endotoxin | 0.3 | 120 ± 5.61 |
| serum | 0.6 | 157 ± 3.50 |
| 2.0-hr endotoxin | 0.3 | 182 ± 3.70 |
| serum | 0.6 | 232 ± 12.30 |
| 4.0-hr endotoxin | 0.3 | 75 ± 5.80 |
| serum | 0.6 | 108 ± 2.20 |
| 6.0-hr endotoxin | 0.3 | 48 ± 5.00 |
| serum | 0.6 | 99 ± 4.20 |

[a] Each figure is based on 4 replicate plates and expressed as the mean ± 1 SEM.

Serum colony stimulating activity at these various times was measured and is shown in Table II. Two different doses of each serum sample were measured. The results indicate that the greatest number of colonies were obtained with serum collected at 2 hr after endotoxin injection which corresponds to the time when the granulocytes in the donors were being released into the peripheral blood. Colony stimulating activity could be demonstrated as early as 0.5 hr after injection (when granulocyte numbers were at their nadir) and was still present at 6 hr in the last serum sample (when granulocyte numbers were greatly increased).

*Effect of endotoxin addition directly to the cultures on agar colony forming cells.* Amounts of endotoxin ranging from 0.004 to 4.0 μg were incubated with normal rat serum and added to cultures of rat bone marrow cells (Table III). No enhancement of the colony forming ability to rat bone marrow was shown, and, in fact, the values with endotoxin addition were somewhat lower than the control value corresponding to incubation with normal rat serum alone.

TABLE III. The Effect of Incubation with Endotoxin *In Vitro* on Colony Formation.

| Normal rat serum (ml) | Endotoxin (μg/plate) | Number of colonies/ $10^5$ cells |
|---|---|---|
| 0.20 | | 65 ± 6.4[a] |
| 0.20 | 0.004 | 50 ± 7.6 |
| 0.20 | 0.040 | 54 ± 7.8 |
| 0.20 | 0.400 | 53 ± 4.4 |
| 0.20 | 4.000 | 46 ± 2.0 |

[a] Each figure is based on 4 replicate plates and expressed as the mean ± 1 SEM.

*The distribution of agar colony forming cells from fractionated rat bone marrow cells.* Preliminary wide-range density separation of 8-day-exhypoxic rat bone marrow revealed that rat *in vitro* colony forming cells (CFCs) comprised a relatively homogeneous population of cells whose buoyant density was between 26.5 and 29.0% BSA (Table IV). Layer D (27.0–29.0% BSA) exhibited a response 3–4 times greater than the response of unfractionated bone marrow, while layer C (26.5–27.0% BSA) gave a smaller but still highly significant response when compared to unfractionated bone marrow (Table IV).

A comparison of the different cell types (Table V) found in the various cell fractions (indicated in Table IV) implicates the "transitional cell" class as the category responsible for the numbers of colonies formed. Since not all cells classified as "transitional cells" produced colonies, it is likely that the agar colony forming cell represents a subpopulation of this cell class. The "transitional cells" are characterized by a round, central leptochromatic nucleus with a thin rim of cytoplasm containing varying shades of basophilia (9–11). In size, these cells appear intermediate between small mononuclear cells (small lymphocytes) and the larger "blast" cells (myeloblasts and proerythroblasts).

*Discussion.* The evocation of the CSF after endotoxin administration with subsequent differentiation along the granulocytic pathway suggests that this factor may be a regulator of granulocyte production (12–14). The present study has shown that an injection of endotoxin into male rats resulted in an early elevation in serum CSF activity. By 2 hr after endotoxin in the donor rats, there was an accelerated release of granulocytes into the peripheral blood, and CSF actvity was still detectable. Similar results have been reported in the mouse by Quesenberry *et al.* (12). In addition, they also found that endotoxin itself had no in-

TABLE IV. Effect of CSF on the Number of Colony Forming Cells Obtained from Different Fractions of 8-Day-Exhypoxic Rat Bone Marrow.

| Marrow fractions[a] | Number of colonies/ $10^5$ cells | $P$ |
|---|---|---|
| Unfractionated | 121 ± 7.0[b] | |
| A (17.0–25.5% BSA) | 30 ± 2.5 | <.001[c] |
| B (25.5–26.5% BSA) | 39 ± 1.4 | <.001 |
| C (26.5–27.0% BSA) | 191 ± 7.4 | <.001 |
| D (27.0–29.0% BSA) | 446 ± 16.5 | <.001 |
| E (29.0–31.0% BSA) | 77 ± 6.1 | <.010 |
| F (31.0–35.0% BSA) | 30 ± 1.9 | <.001 |

[a] Each fraction was stimulated with 0.2 ml of 2-hr endotoxin serum.

[b] Each figure is based on 4 replicate plates and expressed as the mean ± 1 SEM.

[c] $P$ values for each fraction are compared to unfractionated value.

trinsic stimulating activity for mouse CFCs. In fact, inhibition of colony formation was observed when bone marrow cells were incubated with the higher concentrations of endotoxin *in vitro*. We have confirmed these results using rat CFCs.

The mechanism by which endotoxin induces colony stimulating activity in serum is unclear. One hypothesis is that endotoxin affects granulopoiesis as a result of interaction with peripheral leukocytes causing them injury with the ensuing granulocytopenia triggering the elaboration of a substance that accelerates the rate of granulocyte production. Peripheral blood cells have been reported to be a source of CSF (15, 16). Although the granulocyte has been implicated as the blood cell source of CSF (16), it would now seem more likely that it is the blood monocyte (17–19). Another possible explanation is that CSF may appear as a result of a direct action on a target organ unrelated to the granulocytopenia observed. Support for this view is derived from the observations of Shadduck *et al.* (20) who administered antineutrophil serum to cyclophosphamide-induced neutropenic recipients who had no detectable CSF levels in their sera. Only after a subsequent injection of antineutrophil serum could they obtain CSF activity.

It is well-known that CSF causes agar colony formation *in vitro,* but the type of cell responsible for these colonies is still being investigated. Various attempts have been made to separate *in vitro* agar colony cells (CFCs) and spleen colony forming cells (CFUs) in mouse hematopoietic tissue (3, 4, 5, 11, 21–23). Although some success has been attained, the considerable size and density heterogeneity reported for both the CFU and the *in vitro* CFC in mice has prevented extensive enrichment and thus morphological identification of the representative population of these cell types. An alternative approach taken by Moore *et al.* (6) was to apply separation techniques in species other than the mouse where stem cell and progenitor cell populations proved more homogeneous. They described the separation of *in vitro* agar colony forming cells from monkey bone marrow and implicated the "transitional" lymphocyte as the cell respon-

TABLE V. Percentage of Cell Types Found in the Various Fractions of 8-Day Exhypoxic Rat Bone Marrow. Differential Counts are Based on a Total of 1000 Nucleated Cells for Each Marrow Fraction.

| Marrow fraction | Destroyed cells | Erythroblasts | Myeloblasts | Promyelocyte | Small mononuclear | Transitional cells | Other cells[a] |
|---|---|---|---|---|---|---|---|
| Unfractionated | 3.60 | 5.00 | 2.60 | 2.00 | 40.80 | 4.80 | 41.40 |
| A (17.0–25.5% BSA) | 18.00 | 52.90 | 3.00 | 1.20 | 7.60 | 2.50 | 12.40 |
| B (25.5–26.5% BSA) | 13.40 | 36.60 | 16.80 | 2.80 | 15.00 | 3.80 | 7.60 |
| C (26.5–27.0% BSA) | 10.40 | 17.80 | 13.20 | 7.00 | 33.40 | 5.80 | 7.00 |
| D (27.0–29.0% BSA) | 7.20 | 3.00 | 8.20 | 4.80 | 54.80 | 13.80 | 8.00 |
| E (29.0–31.0% BSA) | 5.60 | 6.00 | 4.00 | 2.20 | 59.60 | 4.20 | 18.40 |
| F (31.0–35.0% BSA) | 5.20 | 2.20 | 1.00 | 0.80 | 21.60 | 2.40 | 66.80 |

[a] Other cells include plasma cells, macrophages, megakaryocytes, eosinophils, myelocytes, and mature neutrophils.

sible for colony formation. Although the cell separation techniques utilized in the present study have not resulted in completely uniform groups of cell types, the results tend to support the contention of Moore *et al.* (6) that agar colony forming cells are found in a class of cells described as "transitional" in nature and intermediate in size between small lymphocytes and the larger "blast" cells. Since a greater number of colonies would have been expected if all "transitional cells" formed colonies, it was concluded that the agar colony forming cell represents a subpopulation of the "transitional cell" population.

*Summary.* Rat bone marrow cells formed colonies in agar after stimulation by colony stimulating factor (CSF) obtained from endotoxin-injected rats.

Separation of rat agar colony forming cells (CFCs) was performed on a discontinuous bovine serum albumin density gradient (BSA). Cell fractions obtained between 26.5 and 29.0% BSA showed enrichment for rat agar colony forming cells when cultured with CSF. Although the cell separation has not been complete the present studies support the contention that a subpopulation of cells intermediate between small lymphocytes and "blast" cells and resembling "transitional cells" are responsible for the agar colonies produced.

1. Bradley, T. R., and Metcalf, D., Aust. J. Exp. Biol. Med. Sci. **44**, 287 (1966).

2. Metcalf, D., J. Cell. Physiol. **76**, 89 (1970).

3. Haskill, J. S., McNeill, T. A., and Moore, M. A. S., J. Cell. Physiol. **75**, 167 (1970).

4. Moore, M. A. S., McNeill, T. A., and Haskill, J. S., J. Cell. Physiol. **75**, 181 (1970).

5. Worton, R. G., McCulloch, E. A., and Till, J. E., J. Cell. Physiol. **74**, 171 (1969).

6. Moore, M. A. S., Williams, N., and Metcalf, D., J. Cell. Physiol. **79**, 283 (1971).

7. Zucali, J. R., Rakowitz, F., Van Zant, G., and Gordon, A. S., Blood **40**, 966 (abstract).

8. Dicke, K. A., van Hooft, J. I. H., and van Bekkum, D. W., Transplantation **6**, 562 (1968).

9. Yoffey, J. M., "Bone Marrow Reactions." Williams and Wilkins Co., Baltimore (1966).

10. Orlic, D., Gordon, A. S., and Rhodin, J. A. G., Ann. NY Acad. Sci. **149**, 198 (1968).

11. Murphy, M. J., Jr., Bertles, J. F., and Gordon, A. S., J. Cell Sci. **9**, 23 (1971).

12. Quesenberry, P., Morley, A., Stohlman, F., Jr., Rickard, K., Howard, K., and Smith, M., New Eng. J. Med. **286**, 227 (1972).

13. Chervenick, P. A., J. Lab. Clin. Med. **79**, 1014 (1972).

14. Rothstein, G., Hugl, E. H., Chervenick, P. A., Athens, J. W., and Macfarlane, J., Blood **41**, 73 (1973).

15. Chervenick, P. A., and Boggs, D. R., Science **169**, 691 (1971).

16. Robinson, W. A., and Pike, B. L., *in* "Hemopoietic Cellular Proliferation" (F. Stohlman, Jr., ed.), p. 249. Grune and Stratton, New York (1970).

17. Moore, M. A. S., and Williams, N., J. Cell. Physiol. **80**, 195 (1972).

18. Chervenick, P. A., and Lo Buglio, A. F., Science **178**, 164 (1972).

19. Golde, D. W., and Cline, M. J., J. Clin. Invest. **51**, 298 (1972).

20. Shadduck, R. K., and Nagabhusganen, N. G., Blood **38**, 559 (1971).

21. Bennett, M., and Cudkowicz, G., *in* "The Lymphocyte in Immunology and Haemopoiesis" (J. M. Yoffey, ed.), p. 183. E. Arnold Ltd., London (1967).

22. van Bekkum, D. W., Van Noord, M. J., Matt, B., and Dicke, K. A., Blood **38**, 547 (1971).

23. Metcalf, D., Moore, M. A. S., and Shortman, K., J. Cell Physiol. **75**, 181 (1970).

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