Colony Production *in Vitro* by Regenerating Marrow[1] (35063)

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Repopulation of hematopoietic elements in a mechanically depleted segment of rabbit femur is due directly to cells normally resident in bone (1, 2). It appears that the regenerative process is initiated by mesenchymal cells in the connective tissue of the Haversian canals. Conceivably, such cells could serve as progenitors of hemic colony forming cells resident in marrow proper. In the present study, we examined colony forming capacity of the regenerating marrow using soft agar cultures, although there is reason to believe that the *in vitro* colony forming units (CFU) assayed in this way are not the same as the *in vivo* CFU (3–5). Bennett *et al.* (4) have suggested that it may be a granulocyte precursor analogous to the erythropoietin sensitive cell in the red cell series.

Materials and Methods. New Zealand rabbits, age 10 weeks and weighing 2 to 2.5 kg were used as the experimental animal. Bone marrow was removed from a 3-cm segment of the right femur shaft by perfusion with a 5% dextran solution. Details of this procedure are described elsewhere (6). *In vitro* CFU of marrow were assayed in 4 control rabbits and in 35 rabbits at various stages of regeneration after marrow removal. In each experimental group, there were 2–7 rabbits. An aliquot from both the right and left femoral marrow was collected and weighed. The marrow was then dispersed into a known volume of Hanks' balanced salt solution. Total nucleated counts were made on a hematocytometer and smears were prepared and treated with Wright's stain. Five hundred nucleated cells were enumerated for the differential count. In those instances where fewer cells were present on a single slide, all of the cells were counted.

The method used for culturing the bone marrow was a modification of the one developed by Bradley and Siemienowicz (7). Culture medium consisted of Eagle's minimal essential medium (MEM) with Earle's salts supplemented with MEM vitamins, sodium pyruvate, and nonessential amino acids, 10% fetal calf serum, and 10% trypticase soy broth. Rabbit serum was used in place of a feeder layer to stimulate growth. The constituents for the medium with the exception of soy broth were obtained from Grand Island Biological Company. Large quantities of fetal calf and rabbit sera were procured at one time. Upon arrival at the laboratory the sera were thawed, packaged in small aliquots and refrozen. They were thawed again just before use. When a second batch of serum was introduced, duplicate cultures were assayed for CFU in order to establish that each batch supported the same amount of growth.

Agar was prepared in 0.6 and 1% aqueous solutions at 40°. The cultures consisted of two layers. The basal layer contained 2 parts double strength medium and 2 parts 1% agar at 40° to which 1 part rabbit serum was added. A 2-ml aliquot of this mixture was pipetted into 35-mm petri dishes (Falcon Plastics) and allowed to gel. The top layer consisted of a known number of bone marrow cells suspended in 0.2 ml of Hanks' balanced salt solution which was added first to 1 ml of double strength medium and then to 1 ml of 0.6% agar at 40°. One-ml aliquot of this mixture was layered on top of the basal layer and allowed to gel. Each plate contained either 1.25, 2.5, 5, or 10 $\times$ 10^4 bone marrow cells. Two to six plates of each dilution of bone marrow cell suspension were

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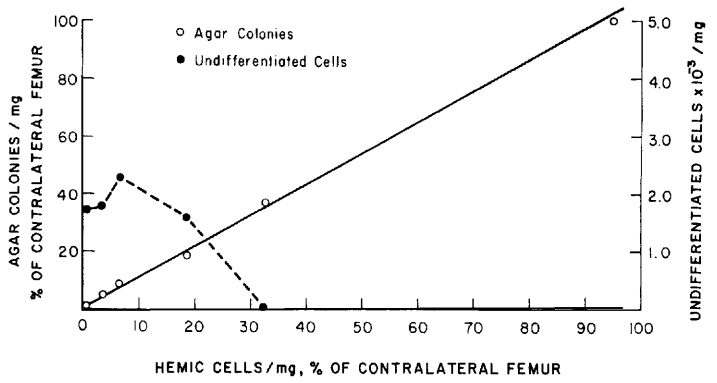

FIG. 1. Agar colonies of regenerating marrow.

prepared. The cultures were incubated at 37° in a 5% CO_2 incubator in the manner described by Abraham *et al.* (8). Colonies of 50 or more cells were counted at 14 days with a dissecting microscope at 20×. The number of colony formers per unit weight of regenerating marrow was related to the number in the contralateral femoral marrow.

Results and Discussion. The colonies observed in soft agar cultures consisted of mononuclear cells with no recognizable granulocytic characteristics. Marrow from four normal rabbits contained 77 ± 8 CFU/mg, 1 mg containing 6.1 ± 5 × 10^5 hemic cells exclusive of polymorphs. The ratios for CFU and for cellularity in right to left femoral marrow were 1.0 ± 0.5 and 1.1 ± 0.6, respectively. Marrow from 35 contralateral femurs of rabbits with a locally regenerating femoral marrow yielded 77 ± 6 CFU/mg (equivalent to 5.4 ± 3 × 10^5 cells exclusive of polymorphs). When the values for normal marrow were compared with those for the contralateral marrow in the experimental animal, the ratios were also 1.0 for CFU and 1.1 for cellularity. Previous studies of a large series of normal rabbits and rabbits with locally depleted femora also demonstrated that the depopulation procedure did not significantly influence the marrow of the contralateral femur in relation to both total cellularity and tritiated thymidine uptake (6).

Although agar colony-formers may be detected as early as 4 days after femur depopulation, such cells represent less than 1% of the control value until the second week. The number of *in vitro* CFU increases approximately exponentially and reaches the normal level by the fifth week. It is noteworthy that this recovery pattern corresponds very closely to that seen for the restoration of recognizable hemic cells. As shown in Fig. 1, there is a linear relationship between CFU content and cellularity of regenerating marrow with a slope which is essentially unity. Since there is little difference in the overall rate of recovery of myeloid and erythroid elements (6), these data do not permit a correlation of *in vitro* CFU restoration wth a specific hemic cell class.

If the *in vitro* CFU is indeed a granulocyte progenitor, it follows from the relationship between CFU content and hemic cellularity that recovery of the normal complement can occur without a perceptible delay in maturation. A similar picture is seen in the evolution of maturing hemic cells consequent to marrow removal (6). Preliminary data suggest that recovery of the marrow CFU as assayed by the conventional spleen colony method is also directly correlated with recovery of hemic cellularity.[2] The nature of the relationship between the *in vitro* CFU recovery and hemic cellularity is clearly indicative of a sustained influx from ancestral cells.

It is known from our previous work (2) that a substantial number of undifferentiated cells appear in the organizing blood clot soon after marrow removal. Autoradiographic studies with tritiated thymidine suggest that

[2] Phillips, T., Maloney, M. A., and Patt, H. M., unpublished data.

such cells are derived from the adjacent osseous tissue during the first week after depopulation. It is clear from Fig. 1 that the recovery of agar colony formers is not directly correlated with the presence of undifferentiated cells. However, the data are consistent with the hypothesis that the former could be a derivative of the latter.

Summary. A study has been made of agar colony forming capacity of marrow during regeneration after localized depletion. From the second week of regeneration agar colonies (*in vitro* CFU) of marrow are readily observed. *In vitro* CFU then increase linearly with hemic cellularity with a slope of about one. If the *in vitro* CFU represents a granulocyte precursor, the data suggest that recovery of these cells requires a sustained influx from ancestral cells.

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