IDENTIFYING CHARACTERISTICS OF THE HAEMATOPOIETIC PRECURSOR CELL

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SUMMARY

Studies are reported utilizing the known ability of homologous marrow cells to prevent death in otherwise lethally X-irradiated mice by 'colonizing' their spleens. The proportion of marrow cells functioning as progenitor cells attains a peak value during post-hypoxic polycythaemia. On the third day post-hypoxia, bone marrows of mice were harvested and prepared for density-gradient centrifugation. The relatively pure fractions of marrow cells were concentrated on the basis of their density and directly injected into the surgically exteriorized spleens of mice which had received an otherwise lethal dose of X-irradiation 48 h previously. Animals were sequentially killed from 15 min to 8 days later and spleens were prepared for examination by light and electron microscopy.

Eighty to 95% of the light-density fraction ($p = 1.0200-1.0440$) was composed of large mononuclear cells characterized by a leptochromatic nucleus with pachychromasia at the nuclear envelope, one or more prominent nucleoli, sparse endoplasmic reticulum, a moderate number of mitochondria, a conspicuous Golgi zone and a centrosome in the region of a nuclear indentation. Cells of this light fraction, devoid of erythroid elements, were competent in colonizing spleens (erythrocyte, granulocyte and megakaryocyte series) in contrast to the cells present in fractions of greater density.

The name 'haematopoietic precursor cell' (HPC) is tentatively used in describing this cell which is discussed in the light of some of the proposed models of haematopoiesis.

INTRODUCTION

The haematopoietic stem cell, despite numerous attempts at determining its precise identity, has remained a subject for speculation. Calculations based on cell kinetics predict that it represents only 3–5% of nucleated marrow cells (Lajtha, 1962, 1964, 1967; Kretchmar, 1966). An uncertainty principle has compounded the problem, for recognition of the stem cell involves the detection of its descendants. By definition progenitor cells must differentiate: hence the cells under scrutiny (i.e. haematopoietic stem cells) are necessarily lost in the process of their detection (Lajtha & Oliver, 1962; Lajtha, Oliver & Gurney, 1962).

The development of spleen-colonizing techniques (McCulloch & Till, 1960, 1961, 1962) has opened a new approach. Cells which colonize the spleen possess the 2 fundamental properties of progenitor cells: (1) the ability to give rise to differentiated descendants, and (2) the capacity for self renewal.

Under periods of stress the size of the precursor-cell population may increase in

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response to stimuli to differentiate. For example, hypoxia causes the production and release of increased amounts of erythropoietin which in turn directs the erythropoietin-committed precursor elements into cells of the erythroid series (Gordon, Cooper & Zanjani, 1967). The precursor compartment must continually expand to meet the depopulation of its numbers as a consequence of their differentiation, while simultaneously an augmented number of multipotential precursor cells must be maintained. When the stimulating factor is removed (e.g. return of an animal from 0.4 to 1.0 atm. (40.5-101.3 kN m⁻²)), the relative size of the precursor population continues to increase for a period of 3-6 days (M. J. Murphy & A. S. Gordon, unpublished).

The present paper describes experiments in which marrow cell populations, separated by density gradient centrifugation, were assessed for their ability to prevent death in otherwise lethally X-irradiated mice by ‘colonizing’ their spleens. That cell fraction which was effective in this regard was then studied and its progeny were examined by light microscopy and electron microscopy.

MATERIALS AND METHODS

Marrow collection

Groups of 100 CF-1 virgin female mice (Carworth Farms), 23-28 g in weight, were exposed to discontinuous hypoxia of 0.4 atm. (40.5 kN m⁻²) of air (simulated 21,000 ft (6.4 km) above sea level) for 5 days. Three days after return to atmospheric pressure (101.3 kN m⁻²) they were killed by cervical dislocation. Marrow was flushed from femora and tibiae with cold Hank’s Balanced Salt Solution (BSS, Grand Island Biological Supply) into siliconized 15-ml test tubes. One ml of BSS was used for each bone to assure an adequate cell dilution. The marrow cells were suspended with siliconized, fire-polished Pasteur pipettes, passed through a fine stainless steel mesh grid (Millipore Filter Corp.) to remove bone spicules and large cellular clumps, and then kept at ice-bath temperature. Coverslip smears of cell samples suspended in serum were studied with light microscopy, and other samples were prepared for electron microscopy as described subsequently (p. 25).

Density-gradient centrifugation

A modification of the method described by Niewisch, Vogel & Mattioli (1967) was used. In brief, 1-4 x 10⁸ nucleated marrow cells in a volume of 2-3 ml BSS were layered on 30-ml linear gradients, with a density range of 1.0115 to 1.0860, prepared from aqueous solutions of Ficoll (Pharmacia Fine Chemicals) containing 0.1 M NaCl, 0.022 M KCl, and 0.008 M sucrose. The pH was adjusted to 7.1 with NaHCO₃ and the preparations were centrifuged at 11,000 rev/min for 20 min in a Spinco SW 25-1 bucket rotor at 5 °C.

After centrifugation 1-ml fractions were collected, through a needle introduced into the base of the tube in such a way as to avoid the pellet of cells formed during centrifugation, into siliconized glass test tubes kept at 4 °C. Densities were obtained from refractive indices measured at 589 nm with a Zeiss-Abbe refractometer at room temperature. The relations among refractive index, density, and concentration of Ficoll have been determined by Leif & Vinograd (1964) and by Bishop & Prentice (1966). As measurements of the densities of Ficoll solutions by pycnometry yielded results which agreed within experimental error with those of Leif & Vinograd (1964), their expression relating refractive index and density was used. Nucleated cells in each fraction were enumerated by haemocytometer. The presence of up to 5 x 10⁶ nucleated marrow cells per ml did not alter the refractive index of the Ficoll solutions.

Cells to be injected were washed with BSS to remove Ficoll and were concentrated to the appropriate dilution immediately before splenic injection.
X-irradiation

CF-1 virgin female mice the same age as those used as marrow donors were employed as irradiated recipients. Radiation was generated by a 250-kV Picker machine operating at 15 mA and 230 kV. A 0.5-mm copper filter was used with 1.0 mm of aluminium, and the exposures were measured in air with a Victoreen condenser meter at the position occupied by the centre of the animal’s body. The dose-rate averaged 88 R (2.26 x 10^-2 C kg^-1) per min at 45 cm. Total exposure time to the X-ray beam was 10 min and 12 sec; hence a total of 900 R (2.32 x 10^-1 C kg^-1) was delivered to the entire body. This proved to be an LD100 dose.

Splenectomy

On the basis of overall differences in cell type (see Results), fractions from density-gradient separations were pooled into a 'light fraction' corresponding to a density range of 1.0200-1.0440 and a 'heavy fraction' with cells of a density range of 1.0650-1.0765.

Forty-eight hours before splenic injection 75 mice were X-irradiated as described. This dose reduced the development of endogenous splenic haematopoietic colonies in non-injected control mice to an average of fewer than 0.05 colonies per spleen at 10 days post-irradiation. Each of these 'haematopoietically sterile' mice received an intrasplenic injection of 1 x 10^7 cells from either of the 2 pooled cell fractions, with sham-operated non-injected mice serving as irradiated controls. The intrasplenic rather than the intravenous route of injection was chosen to maximize the seeding and recognition of potential stem cells within the spleen and minimize their systemic loss in non-haematopoietic tissue.

Splenic injection

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Spleens of recipient irradiated mice were exposed aseptically by laparotomy under ether anaesthesia. Cells were injected in a volume of 10 μl of BSS 1 mm deep on the splenic inferolateral surface through siliconized glass microneedles (100 μm internal diameter at the tip). Volume of injected suspension was controlled by a Manostat digipet with an accuracy of 0.2 μl. The abdominal incision was closed by peritoneal suture and external wound clamps.

Representatives of each group (i.e. light-fraction injected; heavy-fraction injected; and sham-operated non-injected controls) were killed according to the following schedule; 15 min; 6, 18, 24 h; 2, 3, 5 and 8 days after splenic injections. The spleens were immediately excised, freed of fat and vascular connective tissue, and cut into 3-5 mm cubes. These were placed into chilled 1% solutions of glutaraldehyde in 0.1 M phosphate buffer (pH 7.4) containing 1% sucrose and stored overnight at 4 °C.

Microscopy

The cubes of fixed spleen tissue were washed in buffer, post-fixed for 1-3 h in 1% OsO_4 containing 1% sucrose in phosphate buffer (pH 7.4), and finally washed twice in distilled water containing 1% sucrose. Tissue dehydration and embedding in Epon 812 were carried out by the method of Luft (1961). An LKB Ultratome and diamond knife were used to cut a series of single alternate thick and thin sections according to Orlic, Gordon & Rhodin (1968). The thick sections were placed on glass slides and examined by light microscopy. The thin sections were mounted on Formvar-coated 75-mesh copper grids, stained with uranyl acetate and lead citrate (Reynolds, 1963), and examined in a Siemens Elmiscope 1 A at 80 kV. Samples of pre-injection cells were prepared and examined in analogous fashion.

RESULTS

Light microscopy of donor marrow

Three-day post-hypoxic mouse marrow at 3 days after cessation of hypoxia was nearly devoid of early erythroid precursors. Late normoblasts comprised up to 4% of nucleated cells, but erythroid cells earlier than this stage were not present. A significant observation was the considerable number of large cells (Fig. 3) with a diameter
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of 10–18 μm and with nuclei measuring 8–15 μm. Cytoplasmic basophilia was variable, but the nuclear chromatin of these large mononuclear cells was homogeneously fine. The presence of one or more nucleoli was common. This cell type accounted for 18–30% of the total nucleated cells. Phase-contrast microscopy of these elements revealed a well-defined centrosome, noted by earlier workers (Weidenrich, 1909; Wallgren, 1911) as a characteristic of lymphoid cells. While the cytoplasm of most of these elements was devoid of granularity, 3–5% contained 0.5–2 μm azurophilic granules randomly scattered throughout the basophilic cytoplasm. Michaelis & Wolf (1902) were the first to have reported such granularity in small but more frequently in large lymphoid cells of the peripheral blood, and Niewisch et al. (1967) have made a similar observation in mouse marrow cells.

Thirty to 44% of the donor marrow cells was composed of cells in varying stages of granulocytopoiesis, the predominant type being polymorphonuclear leukocytes. Megakaryocytoblasts and more mature forms of the megakaryocyte line were seen in all stages of maturation. Small lymphocytes and broken cells, unclassifiable because of nuclear damage, made up the remainder.

Electron microscopy of donor marrow

Ultrastructural analysis of 3-day post-hypoxic mouse marrow confirmed the light-microscopic observations just described: no early erythroid precursors were seen and only a few late normoblasts were in evidence (Figs. 1, 2). Electron micrographs of the large undifferentiated mononuclear cells already observed with light microscopy are shown in Figs. 4, 5.

Those cells observed in mitosis in marrow prepared for light or electron microscopy showed none of the morphological characteristics of granulocytes or megakaryocytes but possessed the cytological features of the large, mononuclear cells just described (Fig. 6).

Electron microscopy of fractions collected after density-gradient centrifugation.

Three fractions of cells will be described: the 'light fraction' with a density range of 1.0200–1.0440; the 'heavy fraction' with a density range of 1.0650–1.0765; and the pellet of cells at the bottom of the centrifuge tube.

The majority of cells of the light fraction (Figs. 7–9) were the large mononuclears described above and small, round pachychromatic cells resembling peripheral blood small lymphocytes. Five to 12% of the light fraction was made up of recognizably differentiated plasma cells and granulocytes (metamyelocytes and polymorphonuclear leukocytes). Cytological indications of damage were minimal.

The heavy fraction was composed mainly of cells in the granulocytic series in all stages of maturation (Figs. 10, 11). Small lymphocytes were present, and the large mononuclear elements already described made up roughly 10% of the fraction.

The gradient pellet was composed of all cell types previously described as well as reticulocytes, adult erythrocytes, connective tissue cells which had escaped the steel-mesh preparatory filtration, cellular debris, and naked nuclei (Figs. 12–14).
Electron microscopy of injected spleens and control spleens

Spleens of sham-operated, non-injected control animals. The spleens of mice which had received $2.32 \times 10^8 \text{ C kg}^{-1}$ of X-irradiation 48 h previously showed marked degeneration of haematopoietic cells, but the connective tissue framework appeared relatively radio-resistant. Numerous macrophages (Figs. 14, 15), endothelial lining cells, and focal aggregations of plasma cells were noted (Figs. 16, 17). Splenic blood vessels remained intact and the endothelial lining cells appeared normal.

Hence, at 48 h after whole-body irradiation the spleens of non-injected, control mice resembled a graveyard of dead and dying haematopoietic cells. All the non-injected control mice died within 14 d after X-irradiation.

Spleens of animals injected with light-fraction cells. It was easy therefore to distinguish injected, healthy haematopoietic cells from their necrotic surroundings. Fifteen min post-injection, the light-fraction cells, readily visible, had not yet migrated from the site of injection. By 6–18 h the splenic, and less likely the systemic, circulation had either distributed these cells or they themselves had moved to all regions of the spleen. Little differentiation was apparent at 6 h; if anything, the degree of nuclear leptochromia of the large mononuclears had increased (Fig. 18). At 18 h, their cytoplasm contained polyribosomes, and most were in intimate association via plasmalemmal interdigitation with neighbouring pseudopodal extensions of macrophages (Fig. 19). At this time the first recognizable elements of the erythroid series were observed. Proerythroblasts were usually found in clusters, most of which were closely associated not only with each other but with macrophages as well.

As noted previously by Orlic et al. (1968) in the erythropoietin-stimulated polycythaemic mouse spleen, and by Schjeide, McCandless & Munn (1964) in nucleated erythroid precursors of chick embryos, proerythroblast maturation into the erythroblast series was heralded by marked chromatin clumping, a reduction of the Golgi zone, and a diminution in the size of the nucleolus. By 48 h after splenic injection of the light-cellular fraction, orthochromatic erythroblasts, characterized by mitochondrial aggregation in a perinuclear array, were observed. At 3 and 5 days after injection of the cells, the characteristic progression of erythroid maturation had occurred (Fig. 20). Splenic examination 8 days after light-fraction injection revealed a tissue in an exponentially increasing proliferative phase; virtually all haematopoietic cell elements were in abundance throughout the now grossly hypertrophied spleen. Due to the great number of cells injected (i.e. $1-4 \times 10^9$) no discrete splenic 'colonies' were observed, rather the entire organ was actively engaged in haematopoiesis.

Evidence of granulocytopoiesis was first seen in spleens which had been injected with light-fraction cells 18 h previously. Developing myeloblasts, in contrast to injected large mononuclears, demonstrated cytoplasmic inclusions in the form of electron-dense, membrane-bounded vacuoles of approximately 0.5 μm in greatest diameter (Fig. 21). Chromatin clumping not unlike that of erythroid cells was also noted in metamyelocytes. These differentiated elements were observed to be grouped in clusters of 4 or more cells, all of which appeared to be in the same stage of maturation and proliferation.
Megakaryocytoblasts were seen 18 h after injection of light-fraction cells. The size of these large elements and their cytoplasmic vacuolization rendered them the most easily identifiable haematopoietic cell.

Ninety-two per cent of the irradiated mice which received light-fraction cells survived 30 days or longer: this is a measure of the cells' haematological competence.

Spleens of animals injected with heavy-fraction cells. Electron microscopy of spleens of mice which received heavy-fraction cells revealed marked differences compared with spleens injected with light-fraction cells. At 15 min and 6 h post-injection, large areas of granulocytic cells were seen close to the injection site. Small numbers of normal polymorphonuclear leukocytes were noted in divergent areas of the spleen at 18 h, but no evidence of normal haematopoiesis was observed. In the spleens studied, no normal haematopoietic cells were apparent after 24 h, and the general appearance of this tissue resembled that of the control non-injected spleens. It is of interest to note, however, that 8% of the mice injected with heavy-fraction survived 30 days or longer. Although this radiation-survival percentage just meets the limits of radiobiological significance, it seems likely that the mononuclear cells within this fraction, serving as progenitor cells, were responsible.

DISCUSSION

Evidence presented in this report strongly suggests that the so-called 'stem' cell is, under the conditions of these studies, a large (10—18 μm in diameter) mononuclear cell with the following morphologic characteristics. Nuclear chromatin is fine (leptochromatic), with some peripheral condensation. There are one or more prominent nucleoli. The cytoplasm shows a moderate number of mitochondria, sparse endoplasmic reticulum, abundant monoribosomes, and a substantial Golgi apparatus and centrosome near the nuclear membrane which is sometimes indented. In situ these cells, while they may assume a number of shapes to conform to their surroundings, usually have a smooth plasma membrane which only rarely displays projections longer than 0.1 μm. Lipid droplets and multivesicular bodies are absent, but filamentous protein fibres, not unlike spindle protein, may be present. Mitotic figures are frequent. The name 'haematopoietic precursor cell' (HPC) seems appropriate.

Light-microscopy studies by Yoffey et al. (1968) have indicated a cell, which if not identical to, then very closely resembles the HPC, and which they refer to as the 'transitional lymphocyte'. Although HPC have the undifferentiated characteristics of lymphocytes which have enlarged and presumably have become biochemically active, our study provides no definitive evidence for or against this interpretation.

Niewisch et al. (1967) have described a 'large cell with a rather homogenous overall appearance...The cytoplasm is intensely basophilic'. They named these elements 'B' cells, for basophilia. Fractions of mouse marrow containing 20% 'B' cells were more efficient in repopulating lethally irradiated mouse spleens than other bone-marrow-cell fractions. The brief description they provide resembles closely that of cells identified by this study as HPC. In addition, an ultrastructural study of Rifkind, Chui & Epler (1969) of hepatic haematopoiesis in the foetal mouse describes cells that...
the authors refer to as 'presumptive haemocytoblasts'. These cells and HPC have similar characteristics.

Morphologically, all HPC were indistinguishable, but the question remains whether there are subtle differences, both chemical and physical, marking one cell for maturation in the red cell series, another for maturation in the granulocyte series, and another seemingly identical cell as a megakaryocytic precursor. In this regard, McCulloch & Till (1963) have proposed a model based on the assumption that the true haematopoietic stem cell (they refer to this as the colony-forming cell or unit, CFU) may follow either of 2 possible pathways. The first, self-renewal, results in the production of new stem cells. The second, differentiation, culminates in the development of cells adapted to specialized function. Thus the stem cell might become an erythropoietin-committed cell, which, when presented with the proper haematopoietic environment and erythropoietin stimulation, will differentiate into a proerythroblast. Morphologically the difference between the stem cell and the erythropoietin-committed cell has not been delineated, and electron microscopy may not be sufficient to distinguish it.

Another view might depict the stem cell, in its cell cycle, progressing through differing phases of humoral receptivity (Kretchmar, 1966). Here the cycle assumes primary importance, for only at specified times would humoral signals find a responsive cell. It is obvious that numerous interpretations of what could be basically one phenomenon are possible. Regardless of the fine points of biochemical genetics remaining to be determined, the results presented here identify, on a morphological and developmental basis, a population of haematopoietic progenitor cells which gives rise to erythrocytes, granulocytes, and megakaryocytes.

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REFERENCES


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Figs. 1–3. Electron microscopy (Figs. 1, 2) and light microscopy (Fig. 3) of 3-day post-hypoxic marrow containing reticulocytes (r), late normoblasts (n) and pale-staining mononuclear elements (arrows) which are often seen in mitosis. These are implicated as the haematopoietic precursor cell. × 5200, 5200 and 3100, respectively.
Figs. 4, 5. Electron microscopy of 3-day post-hypoxic marrow. The large mononuclear cells are characterized by a leptochromatic nucleus with pachychromia developing along the perimeter of the nuclear envelope which is often indented, one or more prominent nucleoli, sparse endoplasmic reticulum and a modest number of mitochondria. ×11500 and 8400, respectively.

Fig. 6. A primitive cell seen in division. Note the undifferentiated nature of the extra-chromatin material. ×14000.
Haematopoietic precursor cell
Fig. 7. The cellular constitution of the 'light fraction' after density gradient centrifugation. The predominant cell category is the primitive mononuclear type here shown with a granulocytic (g) 'contaminant'. × 6000.

Figs. 8, 9. The prominent nucleoli (arrows), diffuse chromatin, and undifferentiated cytoplasm characterize the undifferentiated mononuclear cells of the 'light fraction'. × 11700 and 12600, respectively.
Haematopoietic precursor cell
Figs. 10, 11. Cells found in the ‘heavy fraction’ after density-gradient centrifugation are primarily those of the granulocytic series. × 8500 and 49,000, respectively.
Haematopoietic precursor cell
Figs. 12, 13. Electron microscopy of the gradient pellet displays clumped cells in large numbers, granulocytes (g), megakaryocytes (m), connective tissue cells and cellular debris. Both × 4400.
Figs. 14, 15. Splenic parenchyma 48 h after 900 R ($2.32 \times 10^{-3}$ C kg$^{-1}$) of X-rays. The tissue is devoid of haematopoiesis and filled with macrophages (arrows) containing phagocytic vacuoles. $\times 4400$ and 4000, respectively.
Haematopoietic precursor cell
Fig. 16. Plasmacytes in various degrees of maturity found in the spleen of a mouse which received $2.32 \times 10^{-1}$ C kg$^{-1}$ of X-rays 48 h previously. Part of a macrophage (ma) is also seen. $\times 5000$.

Fig. 17. A higher magnification of 2 plasmacytes from tissue prepared as described in Fig. 20. Note the well defined and normal Golgi zone (arrows). $\times 6600$. 
Haematopoietic precursor cell
Figs. 18, 19. Haematopoietic precursor cells (h) 18 h after injection of 'light fraction' into the spleen. The cells retain their primitive morphology and cytologic integrity. They are seen in close association with neighbouring macrophages (ma). x 8000 and 10800, respectively.
Haematopoietic precursor cell
Fig. 20. At 3 days after splenic injection of 'light-fraction' cells erythroid colonies surrounded by phagocytic macrophages (ma) and connective tissue elements are noted. This is an example of an orthochromatic erythroblast (e) nest, all of the same degree of differentiation. × 2300.

Fig. 21. At 3 days after injection of 'light-fraction' cells an immature but normal eosinophilic granulocyte (eo) and a plasmacyte (p) are observed. × 5000.
Haematopoietic precursor cell