Human Marrow Erythropoiesis in Culture. I. Characterization of Methylcellulose Colony Assay

By Makio Ogawa, Richard T. Parmley, Harvey L. Bank, and Samuel S. Spicer

We examined the morphological and functional characteristics of human marrow erythrocytes cultured with a recently developed methylcellulose colony assay technique. Erythrocytic cells in various stages of development were observed, and a significant degree of maturational synchrony within individual colonies was noted. By light microscopy, colonies consisting of late normoblasts appeared compact, had an orange hue attributable to their hemoglobin, and demonstrated pseudoperoxidase activity, whereas colonies composed of early erythroblasts grew less compact or in clusters of smaller cell aggregates and showed no reddish tinge. Colonies possessing intermediate features were also observed. Maturational synchrony of individual colonies was confirmed using transmission and scanning electron microscopy. The ultrastructure and cytochemistry of most immature cells were normal. The mature erythrocytes, however, were severely microcytic and hypochromic and contained one to several Heinz bodies. These defects in the cytoplasmic maturation of erythrocytes corresponded with impaired granulocytic maturation in culture, which we observed previously, and suggest environmental or nutritional defects in culture. Linearity of the method was confirmed using five normal bone marrows. Erythropoietin dose-responses observed in ten normal marrows were comparable to the previously reported results and revealed significant variation in individual plating efficiencies.

Over the past decade, the availability of semisolid culture techniques for human granulocytes has aided our understanding of the dynamics of granulocytic development in normal and pathologic conditions. Recently, similar clonal cell culture assays using plasma clot or methylcellulose were introduced for culture of human erythropoietic precursors. Precise characterization of the cells grown in these systems was a prerequisite to the application of these assays in the study of normal and pathologic erythropoiesis. This communication describes cytochemical and ultrastructural characteristics of human marrow erythropoietic cells cultured in the methylcellulose assay and defines normal kinetic values of colony formation.

Materials and Methods

Bone Marrow Culture Assays

After an informed consent was obtained, marrow cells were aspirated from the posterior iliac crest of healthy volunteers with standard sterile technique. The volunteers consisted of students and house-staff members and averaged 31 yr of age. Marrow cells were collected in 6-ml Falcon...
plastic tubes containing heparin without preservatives (Connaught Laboratories, Willowdale, Ont., Canada). The marrow buffy coat cells were washed twice and were separated from adherent cell populations by incubation for 3 hr in 100-mm Falcon plastic tissue culture dishes as described by Messner et al.\textsuperscript{3} Nucleated cells in desired concentration were plated into 35-mm Lux standard non-tissue culture dishes (Flow Laboratory, Rockville, Md.) in a mixture containing \( \alpha \)-medium (Flow Laboratory), 0.8\%, methylcellulose (Fisher Scientific Co., Norcross, Ga.), 30\% fetal bovine serum (Flow Laboratory), 1\% bovine serum albumin (Calbiochem, San Diego, Calif.), and a desired concentration of a step III preparation of sheep plasma erythropoietin containing 0.2-0.5 U/mg (Connaught Labs). The bovine serum albumin had been deionized and buffered as described by Tepperman et al.\textsuperscript{1} Colonies and their cellular constituents were examined after incubation for 7-10 days at \( 37^\circ \text{C} \) in 5\% \( \text{CO}_2 \) in air. At this concentration of \( \text{CO}_2 \), the pH of \( \alpha \)-medium was kept at 7.4.

**Light Microscopy**

Colonies in the dishes were observed either directly on an inverted light microscope through a green or blue filter or after staining for red cell pseudoperoxidase activity by incubating with the substrate 3,3'-diaminobenzidine (DAB). This staining utilized the technique of Graham and Karnovsky\textsuperscript{4} with a slight modification. The substrate medium contained 3 mg of DAB dissolved in 10 ml of 0.05 M Tris-HCl buffer, pH 7.6. Two milliliters of 3\% hydrogen peroxide solution were added to the DAB solution just prior to use, and approximately 0.5 ml of the mixture was layered over the methylcellulose culture from a Pasteur pipette. Dishes were incubated at room temperature for about 15 min and then viewed on an inverted microscope. In addition, individual colonies were picked from the dish with a fine Pasteur pipette and stained by Wright's stain or by supravital staining with crystal violet for Heinz bodies.\textsuperscript{5}

**Transmission Electron Microscopy**

Individual whole colonies were prepared for transmission electron microscopy by a modification of the technique described by Zucker-Franklin and Grusky.\textsuperscript{6} Approximately 1.0 ml of the fixative, consisting of 3\% glutaraldehyde in 0.1 M cacodylate buffer, pH 7.4, was added to the culture dish and incubated for 20 min at room temperature. Two per cent agar (Difco Laboratories, Detroit, Mich.) was added to bring the final solution to one part methylcellulose, one part fixative, and one part agar. The solidified mixture forming an agar disc was held overnight at 4\(^\circ\text{C}\). The specimen was then rinsed in 0.1 M cacodylate in 7.5 g/100 ml sucrose buffer and stored overnight. In addition, bone marrow colonies in culture medium were fixed in an equal volume of 3\% glutaraldehyde buffered with 0.1 M cacodylate buffer and then centrifuged into a pellet at 500 g for 3 min and rinsed. These cells were incubated in DAB substrate medium for demonstration of peroxidase and pseudoperoxidase.\textsuperscript{4} Some specimens were centrifuged and were fixed in a slightly modified\textsuperscript{7} Komnick solution\textsuperscript{8} for localization of antimonate reactive cation and in a control fixative containing osmium tetroxide with pyrophosphate in place of pyroantimonate. All specimens except those fixed initially with osmium tetroxide were then rinsed in cacodylate-buffered sucrose and postfixed in 2\% osmium tetroxide at room temperature for 1 hr. The pellets were then routinely dehydrated and embedded in Epon. In the final infiltration step, individual whole colonies were separated under a dissecting microscope from the agar disc and embedded in Epon capsules. Thin sections of morphological specimens were counterstained with uranyl acetate and lead citrate, whereas cytochemical preparations were examined unstained. The sections were examined in a Hitachi HS-8 or AEI-EM 6B electron microscope at an accelerating voltage of 50 kV.

**Scanning Electron Microscopy**

A sufficient volume of the 0.2 M phosphate-buffered saline (PBS) was added to dishes containing the erythrocytic colonies to further solubilize the methylcellulose. The colonies were allowed to settle, and most of the media was decanted. They were then fixed in 2\% glutaraldehyde buffered in 0.1 M cacodylate buffer at pH 7.4 (360 mOs) for 2 hr. The samples were subsequently washed in PBS and postfixed for 2 hr in 2\% osmium tetroxide buffered at pH 7.4 with phosphate. The intact colonies were further washed with PBS and treated with saturated thiocarbohydrazide followed by an additional fixation in 2\% osmium tetroxide. The specimens were then dehydrated through a
Fig. 1. (A) A medium-sized, well-hemoglobinized colony viewed through a colored filter. (B) The same colony stained with DAB in culture. (C) Wright's stain of a similar colony showing normoblasts with condensed heterochromatin. The arrow indicates a non-nucleated mature erythrocyte.

RESULTS

Light Microscopy

The majority of the colonies were small-to-medium sized, containing 50–500 cells and, when viewed through colored filters, showed a distinct orange hue attributable to their hemoglobin (Fig. 1A). Individual cells possessed polygonal contours and appeared tightly aggregated in each colony. The entire colonies stained dark brown to black with DAB (Fig. 1B). The smears of these colonies with Wright’s stain revealed late erythroblasts, i.e., normoblasts which were polychromatophilic or orthochromatic (Fig. 1C). Small numbers of colonies showed no sign of hemoglobin accumulation and had a distinctly different morphological appearance. Cells in these colonies were large, round, and somewhat loosely arranged (Fig. 2A). The colonies occasionally revealed several small aggregates of the latter cells in clusters. Such colonies failed to stain for pseudoperoxidase with DAB and hydrogen peroxide (Fig. 2B), and were composed of early erythroblasts, i.e., pronormoblasts and basophilic normoblasts (Fig. 2C). When these colonies were incubated and examined daily over 14 days, gradual accumulation of hemoglobin in the constituent colonies was observed. In addition, erythroctic colonies possessing intermediate morphological features were observed. Although clear separation from the other
two groups was difficult, the colonies of this group were somewhat larger than those of the more hemoglobinized group and consisted of mixtures of normoblasts at various stages of maturation.

Mature non-nucleated erythrocytes were extremely hypochromic and microcytic. When stained supravitally with crystal violet, these cells revealed the presence of one to a few Heinz bodies in the cytoplasm. A few granulocytic colonies were identified by Wright’s stain of their smears when $10^3$ nucleated cells were plated per dish. These colonies could also be recognized by their characteristic appearance without staining.

Toluidine blue-stained epoxy thick sections of whole erythrocytic colonies revealed 20–150 closely packed cells. Most of these cells in a given colony appeared to be maturationally synchronized, showing a similar nuclear to cytoplasmic ratio and nuclear chromatin pattern. Some colonies contained cells with more heterochromatin, whereas cells found in other colonies appeared less mature with dispersed chromatin. The colonies often disclosed mitotic cells. Both granulated erythrocytic cells (sideroblasts) and nongranulated erythrocytic cells were observed within individual colonies, and no other cell types were identified definitely. Dying or dead cells with vacuolated dense cytoplasm and pyknotic nuclei were also seen.

**Transmission Electron Microscopy**

Early erythroblasts (i.e., pronormoblasts and basophilic normoblasts) generally resembled those described in vivo. These varied from 15 μ to 20 μ in diameter and contained a slightly irregular nucleus with dispersed chromatin...
Fig. 3. The cells in this erythrocytic colony appear to be in a similar stage of development. They lie widely separated over much of the cell surface, but parts of the surface approximate each other closely. The cell surfaces are irregular and reveal many pinocytotic caveolae (lower inset). The cytoplasm of all of the cells contains numerous closely packed ribosomes and polyribosomes (insets). Some cells have round granules (siderosomes) which often contain dense particles resembling ferritin (upper inset). There is only sparse endoplasmic reticulum and Golgi lamellae. The nuclei are often indented and the chromatin dispersed. $\times 5600$ (lower inset $\times 17,400$, upper inset $\times 25,000$).

and one or two nuclei. Occasionally, two and rarely three nuclei were present. The cytoplasm of these cells contained abundant free polyribosomes, a few mitochondria, several vesicles, a few vacuoles, rare segments of endoplasmic reticulum, and a variable number of Golgi lamellae (Fig. 3). Ferritin particles were observed in caveoli and microendocytic vesicles, as well as in granules resembling siderosomes (Fig. 3). There was marked variation in the number of siderosomes in different cell profiles, though most cell profiles contained one to
The cells in this colony appear to be in a later stage of development than those in Fig. 3, as is evidenced by the widely separated clumped ribosomes (inset), and more clumped nuclear chromatin. The cell surfaces are more regular, and the cells are more closely packed. There is little evidence of pinocytic activity. Round granules or siderosomes are present in many of the cells. x 5900 (inset x 17,400).

Late erythroblasts (polychromatophilic and orthochromatic normoblasts) varied from 12 to 17 μ in diameter and contained round nuclear profiles with clumps of heterochromatin (Fig. 4). The cytoplasm appeared denser and contained fewer polyribosomes in these mature cells (Fig. 4). Some cells contained siderosomes and vacuoles. The latter cells could be differentiated from dying cells with pyknotic nuclei by the integrity of the cell membranes. Extrusion of the nuclei was observed in occasional mature cells.

Mitotic cells were observed in colonies of early and late erythroblasts, and generally appeared normal in all mitotic phases. The cytoplasmic features of the mitotic cells corresponded with those of adjacent cells. Fewer mitotic cells were observed in colonies of late erythroblasts.
Fig. 5. This non-nucleated red cell appears extremely hypochromic and contains many dense inclusions. The latter are closely associated with the inner membrane and by comparison with cells stained for light microscopy, were identified as Heinz bodies. These cells were frequently observed in centrifuged specimens and occasionally were seen adjacent to mature erythrocytic colonies. × 12,000.

Non-nucleated erythrocytes measuring 3–7 μ in diameter were occasionally observed within more mature red cell colonies. However, such cells were abundant in centrifuged specimens in which all cells present in the culture were collected. These mature cells appeared irregular and elongated and showed abundant cytoplasmic indentations. The very lucent cytoplasm of these cells contained numerous inclusions measuring 0.02–1 μ in diameter and adhering to the inner membrane (Fig. 5). These inclusions resembled Heinz bodies previously described.²

Pseudoperoxidase activity as evidenced by electron opaque reaction product was observed in many nucleated red cells after incubation with DAB (Fig. 6). This staining was not evident in the cytoplasm of very immature cells. The reactivity in non-nucleated mature red cells appeared to decrease in concordance with the severe hypochromicity of these cells.

Antimonate deposits were distributed normally in the nuclear heterochromatin.² Pyroantimonate reactive cation was not evident in the dense inclu-

Fig. 6. Diaminobenzidine reaction product stains the cytoplasm of this nucleated red cell, which appears to be in a later stage of development as evidenced by the condensed nuclear chromatin and sparse cytoplasmic polyribosomes. The reaction product presumably visualizes pseudoperoxidase of the red cells. × 7000.
sions in non-nucleated red cells, indicating that the inclusions were not nuclear remnants.

**Scanning Electron Microscopy**

The colonies varied in size, but appeared to consist of morphologically homogeneous populations of cells (Fig. 7). The individual cells averaged from 14 to 17 μ in diameter, and often appeared somewhat flattened. The surface was sparsely coated with thin microvilli, but occasionally showed irregular regions lacking microvilli (Fig. 8). The cells themselves appeared to be in close apposition with one another, occasionally showing projections interdigitating between neighboring cells.

**Colony Formation**

The erythropoietin dose-response was investigated by plating 10³ nucleated marrow cells per dish in the presence of varying concentrations of erythropoietin (Fig. 9). Colonies did not form in the absence of erythropoietin. The maximal stimulation occurred at an erythropoietin concentration of 0.5 U/ml,
beyond which there was no further increment in the number of erythrocytic colonies. However, different normal bone marrow specimens varied significantly in their colony-forming capacity, ranging from 5 to 280 colonies, with an average of about 75 colonies per $10^3$ nucleated cells. Linearity of the system was tested on five marrow samples by varying the cell concentration plated in the presence of 1.0 U/ml of erythropoietin. The numbers of erythrocytic colonies correlated well with the numbers of cells plated between $0.5 \times 10^4$ and $4 \times 10^5$ nucleated cells per plate (Fig. 10).
DISCUSSION

Two different techniques of clonal cell culture assays are currently available for the assessment of human marrow erythropoiesis. Tepperman et al. developed a plasma clot assay using a modification of the technique originally described for fetal murine erythrocytic colonies by Stephenson et al. Another assay using methylcellulose was later described by Iscove et al. The latter appears to have an advantage over the former because it requires fewer culture ingredients and allows removal of colonies for examination. Accordingly, we chose the methylcellulose culture method to study human marrow erythropoiesis.

The synchrony and degree of cell maturation in each colony was demonstrated by several techniques. Hemoglobinization, as estimated by viewing through a green or blue filter, corresponded to colony maturation and served to assess the maturity of individual colonies. Those colonies consisting of late normoblasts were well hemoglobinized and appeared as tight aggregates of cells. Colonies containing early erythroblasts were loosely arranged or consisted of clusters of smaller cell aggregates. The differing maturity of these colonies may be due to differences in the times at which growth is initiated in culture. This explanation on the basis of variation in the lag times is unlikely, however, since the immature colonies were generally large, and it is thus necessary to assume cells with very short doubling times. An alternative explanation is that the colony-forming units were at variable stages of development. The observation of mitotic cells with variable cytoplasmic maturation supports this hypothesis. It may be that immature colonies were derived from colony-forming units analogous to the murine “burst”-forming units designated by Axelrad et al. to be progenitors of more mature erythrocytic colony-forming units. Intermediate forms were seen, but clear distinction of the three stages of colony maturation was sometimes difficult. Human erythrocytic colonies seen in methylcellulose culture assay thus appeared to originate from precursors representing a continuous spectrum of early erythrocytic development.

Cells at late stages of development were severely microcytic and hypochromic and contained Heinz bodies. In contrast, the development of the nucleus proceeded more normally. These results corresponded with our previously reported data on human marrow granulocytic cells in culture. In those studies, we reported that, while the maturation of the cell membrane and nucleus appeared normal, the cytoplasmic development, particularly that of neutrophil secondary granules and eosinophil crystalloid granules, appeared to be defective. These studies of the human hemopoietic cells in culture suggests nutritional or environmental defects in the existing culture conditions.

An erythropoietin-dose relationship was established and showed maximal stimulation of colony formation with 0.5 U/ml and no spontaneous growth of colonies without erythropoietin. Results of similar studies reported in the literature have been variable. Moriyama et al. noted significant spontaneous growth of erythrocytic colonies by culturing for 4 days, in contrast to the incubation period of 7–10 days used in our system. However, their source of erythropoietin and method of colony identification were different. Tepperman et al. reported a different erythropoietin dose-response using the plasma clot
culture technique. Our results agree with the original data presented by Iscove et al., in which stimulation with 0.5 U/ml and 1.0 U/ml erythropoietin resulted in similar numbers of erythrocytic colonies.

Short-term primary cell culture of human hemopoietic cells is being extensively applied to the study of physiology and pathology of human hemopoiesis. In this communication, we have defined human marrow erythropoiesis in culture using a combined morphological and functional approach. The results appear to suggest that such effort would be useful also in the study of the pathology of human erythropoiesis. Caution has to be exercised, however, when results are interpreted, since defects exist in the present culture system. Development of a cell culture environment more suitable for human primary cell culture is needed.

ACKNOWLEDGMENT

We are grateful to Ms. Sara S. Keirn, Ms. N. Joanne Wright, Ms. Nancy T. Brissie, and Mr. Earl L. Alston for their technical assistance, and to Ms. Linda S. Todd for her help in the preparation of this manuscript.

REFERENCES

11. Parmley RT, Ogawa M, Spicer SS, Wright NJ: Human erythropoiesis in culture. II. Ultrastructural and cytochemical studies of cellular interactions. (to be published)
Human marrow erythropoiesis in culture. I. Characterization of methylcellulose colony assay

M Ogawa, RT Parmley, HL Bank and SS Spicer