Cytochemical and Morphologic Identification of Macrophages and Eosinophils in Tissue Cultures of Normal Human Bone Marrow

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When normal human bone marrow cells are cultured in soft agar, in the presence of human spleen-conditioned medium, two types of colonies could be recognized by means of the inverted microscope: large colonies consisting of macrophages-monocytes, and small colonies consisting of eosinophils. In the two types of colonies, cells developing from blasts to mature cells could be recognized by their morphologic and cytochemical properties. The identification of macrophage-monocytes and of eosinophils was based on light, phase-contrast, and electron microscopy, the strong positive reaction of α-naphthyl acetate esterase in the macrophage-monocyte, and the positive stain with Luxol Fast Blue, eosin, and peroxidase of the eosinophils.

HEMOPOIETIC PROGENITOR cells from mouse and human bone marrow will form colonies when cultured in agar provided either with a feeder layer or conditioned medium (C.M.). In such conditions, granulocyte and macrophage progenitor cells proliferate in soft agar cultures to form colonies of granulocytes and/or macrophages.

Using different methods of colony growth, human bone marrow cells produced colonies which were composed of granulocytic neutrophils. Human bone marrow cells cultured in C.M., derived from peripheral blood leukocytes, developed into large colonies consisting of small peroxidase-positive cells, small colonies made up of eosinophilic and mononuclear cells, or colonies consisting of granulocytes and mononuclear cells. Cultures of peripheral leukocytes from normal subjects produced colonies of eosinophils, neutrophils, monocytes, and macrophages. Full characterization of cells in distinct types of colonies is essential in order to determine the type of hemopoietic progenitor cell which can be induced to grow and mature under in vitro conditions.

The present work describes the nature of hemopoietic cells in different types of developing colonies, as ascertained with the aid of morphologic and cytochemical methods, when normal human bone marrow cells are cultured in soft agar in the presence of human spleen-conditioned medium.

MATERIALS AND METHODS

Preparation of Conditioned Medium

The conditioned medium was prepared from ten human spleens obtained from patients splenectomized because of traumatic rupture of the spleen. The spleens were cut into pieces, crushed, and...
passed through stainless steel mesh in the presence of Dulbecco's modified Eagle medium (Gibco). The isolated cells were counted, and $10^5$ cells were seeded into 90-mm plastic petri dishes with 10 ml of Eagle medium (E.M.) containing 10% fetal calf serum (F.C.S.) (Gibco). In some dishes the cells were seeded with Eagle medium without F.C.S. These dishes were incubated at 37°C, with 5%–7.5% CO$_2$ in a water-saturated atmosphere. The medium was harvested 4 days after seeding, centrifuged at 1000 g for 10 min, and the supernatant was stored at −20°C. This spleen-conditioned medium was used at a concentration of 50% in the bottom agar layer in the assay for colony formation.

**Preparation of Bone Marrow Cells**

Bone marrow specimens (sternal aspirations) were obtained from 15 patients who required this diagnostic procedure, usually for evaluation of mild anemia or investigation of fever of unknown origin. Material selected for this study was used only when the bone marrow smears studied by means of light microscopy of May-Grünwald-Giemsa-stained smears were recognized as normal.

**Preparation of Plates**

The culture method used was the soft agar gel system for culturing mouse bone marrow, described by Pluznik and Sachs$^1$ and by Bradley and Metcalf.$^3$ In control plates, addition of conditioned medium was omitted, or conditioned medium prepared without F.C.S. was used. During 21 days of culturing, the development of colonies and their morphology was observed under an inverted microscope using magnification of 50.

**Morphologic Studies**

Every type of colony was prepared for morphologic and cytochemical studies after 7, 10, 14, and 21 days of culturing. Selected colonies in each culture were punched out using capillary tubes, 0.8 x 100 mm, and their contents were blown onto clear slides, mixed with inactivated F.C.S. (56°C for 30 min), and allowed to dry in air for at least 3–6 hr.

**Phase Contrast Microscopy**

After mixing the colony with F.C.S. as described above, the mixture was covered with a cover slip and observed under the phase-contrast microscope with magnification of 800.

**Electron Microscope**

Samples of different types of colonies were picked out and blown into a test tube containing 2.5%, glutaraldehyde in 0.1 M cacodylate-HCl buffer (pH 7.2) and kept at 4°C for 2 hr for fixation. It was then washed with cacodylate-HCl buffer, reixed in 1%, OsO$_4$ in cacodylate-HCl buffer, dehydrated, and embedded in Epon 812 as described by Luft.$^10$ Sections were made with an LKB Ultratome, and they were examined with a Philips EM 300 electron microscope.

**Cytochemical Studies**

The staining methods used were May-Grünwald-Giemsa (M.G.G.) and peroxidase for general evaluation and for study of eosinophils.$^{11,12}$ Nonspecific esterase was determined by using α-naphthyl acetate and naphthol AS-D chloroacetate as substrates.$^{13}$ Azo dye coupling methods were used for assay of alkaline phosphatase and acid phosphatase activity.$^{14,15}$ Furthermore, periodic acid Schiff reaction (P.A.S.) was carried out before and after ptyaline digestion,$^{16}$ alcoholic or aqueous toluidine blue stains,$^{12}$ and a modified Sakaguchi reaction$^{17}$ were used. Eosin staining was done after fixation with absolute methanol for 5 min. The staining solution consisting of 1%, eosin in 0.1 N NaOH (pH 11). The staining was performed at 25°C for 20 min.

Luxol fast blue staining for eosinophils was done after fixation with formalin vapor at 25°C for 5 min, subsequent rinsing under tap water for 5 min, and drying. A few drops of the staining solution were placed on the fixed colonies and incubated in a humid petri dish at 25°C for 1 hr. The staining solution consisted of 0.1 g Luxol fast blue in 100 ml of 70%, alcohol saturated with urea.$^{18}$ After incubation, the slides were rinsed under tap water and air dried. Counterstaining was done with Harris hematoxylin for 6 min.
RESULTS

When human bone marrow cells were seeded in the presence of human spleen-conditioned medium with F.C.S., two types of colonies could be recognized morphologically and were regularly seen under the inverted microscope between the seventh and the 21st days of incubation.

In control cultures either without conditioned medium or with conditioned medium lacking F.C.S., no colonies were seen.

One day after seeding, numerous single cells were noted in the upper agar layer. Most of these cells disappeared during the succeeding 4 days. By the sixth day, small colonies began to appear and became well defined on the tenth day. The logarithmic phase of colony growth extended from the sixth to the 14th day of culturing, whereas from the 14th to the 21st day, the cultures increased in size very slowly if at all. Thereafter, colonies began to undergo degeneration.

From the seventh day on, the two types of colonies resembled those shown in Figs. 1 and 2. The larger colonies (Fig. 1) were diffuse and consisted of macrophages. By the 14th day they contained 100–300 cells scattered over an area of 1–3 sq mm. The small, compact, and sharply limited colonies (Fig. 2) contained 50–150 clearly defined cells which were eosinophils.

MORPHOLOGIC AND CYTOCHEMICAL STUDIES

Phase-Contrast Microscopy

Single cells from the large diffuse colonies were seen as large irregular cells (Fig. 3). The nucleus was of variable shape with fine condensations, sometimes kidney shaped or even markedly lobulated. The cytoplasm was irregular and almost ameboid, without clear perinuclear zone, and it occasionally contained vacuoles at the periphery. Many cells were filled with granules of varying sizes. Single cells from the small compact colony were seen as medium-sized cells.
possessing many spherical, highly refractile granules. The nucleus could be hardly made out (Fig. 4).

May-Grunwald-Giemsa Stain

At the seventh day, young cells from both types of colonies were closely similar. The nuclei had loose chromatin, often possessed one to three nucleoli, and the narrow rim of cytoplasm was basophilic. Almost all cells contained clearly discernible azurophilic granules. The characteristic morphologic features of either macrophages or eosinophils appeared from the tenth day on.

Cells growing as large diffuse colonies retained the basophilic cytoplasm even on the 21st day. These cells closely resembled in appearance peripheral blood monocytes or exudate macrophages. Their nuclei were round, oval, or kidney shaped with loose chromatin; the margin of the cytoplasm was irregular and contained vacuoles and many azurophilic granules.

In the small, compact colonies, two stages in the development of eosinophil granulocytes could be differentiated on the basis of their granules. Azurophil granules formed during the promyelocyte stage, while eosinophilic granules appeared only during the myelometamyelocytic stage (Fig. 5). A few azurophil granules remained in some eosinophils at all stages of myelocyte maturation.

In the cytoplasm, the basophilia decreased, and the granules more and more resembled those of eosinophilic granulocytes. Further confirmation of the identity of these cells was obtained by their cytochemical characteristics.

Electron Microscopy

It should be stated that conditions existing in our cultures did not preserve the cellular ultrastructure as perfectly as it would be desired.

Monocytes and macrophages from the diffuse colonies showed a population containing a spectrum of cells of varying degrees of maturity with peripheral ruffled margins, typical pseudopodia, or large number of fingerlike projections at the surface. The cytoplasm contained a variable quantity of rough endoplasmic reticulum, free ribosomes, polysomes, and mitochondria in varying
numbers. The Golgi complex was well developed and situated about the centrosome within the nuclear indentation; numerous vesicles were found, some of which were more electron dense, possibly representing early lysosomes. In large macrophages many characteristic lysosomes fusing with phagosomes and numerous vacuoles of varying size were recognizable (Fig. 6A, B).

The cells in the small colonies contained granules resembling eosinophilic granules. They possessed a spherical or slightly oval shape and were similar to granules found in "early" eosinophils. The characteristic crystalloid structure of granules in mature human eosinophils was not encountered in cultured cells, and only rarely were crystalloid forms observed. A striking phenomenon found in the cells was the relatively low electron density in the core region, as compared with the much more electron-dense matrix. Granules were visible within the phagocytic vacuoles, and they could be readily recognized as the "less dense core" of granules within phagosomes (Fig. 7A and 7B).

CYTOCHEMICAL STUDIES (TABLE 1)

Luxol Staining

This green stain, specific for eosinophils, was positive only in cells from the compact colonies. In young cells from these colonies (7–10 days old), the green color was diffuse and faint. When cells matured, the green stain became intensive and granular. The specificity of the Luxol staining for eosinophils has been corroborated by using peripheral blood and bone marrow smears from normal persons and patients with eosinophilia. Only eosinophilic granules were found to react with the dye by taking on greenish coloration.

Eosin Staining

Only the granules of the eosinophils, young or mature, showed deep red color.

Sakaguchi Reaction of Arginine

Although the morphology of stained cells was poorly preserved, positive staining of the eosinophilic granules was clearcut.

Esterase Activity Using α-Naphthyl Acetate Substrate

Only a few fine granules could be found in the blasts. From the 7th day of culture, the cells from the diffuse colonies exhibited strong esterase activity in
Fig. 6. Electron micrograph of macrophage from a different colony after culture for 3 weeks. Numerous vesicles, Golgi complexes (G), endoplasmic reticulum (ER), myelin figures (MF), mitochondria (M), pseudopods, and a vesicular material containing flocculent material, probably representing indigestible remnants such as bacteria (B) and red cell debris (RD) 

(A) x 18,000.
Fig. 7. Electron micrograph of eosinophilic granulocytes after culture of 14 days. The characteristic granules (arrow) are described in the text. Central core of the granule can be recognized within phagocytic vacuoles as well as myelin figure (MY). (A) x 24,500. (B) x 19,000.
Table 1. Cytochemical Findings in Human Bone Marrow Cells Developing as Colonies in Soft Agar Cultures

| Day of cell culture Morphologic classification of cells (M.G.G.) | Large Diffuse Colonies | Small Compact Colonies |
|---|---|---|---|
| | | 7 | 10 | 14 | 21 | 7 | 10 | 14 | 21 |
| | | "Young" | "Young" | "Young" | "Old" | mature | "Young" | mature | Mature | "Old" | mature |
| Cytochemical staining | | | | | | | | | | |
| Esterase activity: | | | | | | | | | | |
| a-Naphthyl acetate | + | + | + | + | + | t | - | - | - | - | - |
| Naphthol AS-D chloracetate | - | t | - | - | - | t | - | - | - | - | - |
| Peroxidase activity | - | - | - | - | - | t | - | - | - | - | - |
| Peroxidase for eosinophils | - | - | - | - | - | + | - | - | - | - | - |
| Lactoferrin blue | - | - | - | - | - | t | - | - | - | - | - |
| Eosin | - | - | - | - | - | t | - | - | - | - | - |
| Toluidine blue for arginase | - | - | - | - | - | + | - | - | - | - | - |
| Alkaline phosphatase | - | - | - | - | - | t | - | - | - | - | - |
| Acid phosphatase | + | + | - | + | + | + | - | - | - | - | - |
| Toluidine blue (alcohol) | t | + | + | + | + | - | - | - | - | - | - |
| Toluidine blue (water) | - | - | - | - | - | - | - | - | - | - | - |

the form of brown granules scattered throughout the cytoplasm as well as diffuse dark cytoplasmatic staining.

The enzymatic activity increased in intensity with maturation of the cells, indicated by brown clumps, fine granules, and diffuse cytoplasmatic staining (Fig. 8).

In the compact colonies, beginning with the 7th day of culture and continuing through subsequent stages, no staining was detected.

**Esterase Activity Using Naphthol AS-D Chloracetate Substrate**

With this substrate a weak enzymatic activity was found in both types of colonies. It was expressed as reddish brown isolated granules in some cells.

**Alkaline Phosphatase**

No enzymatic activity was demonstrated in any of the cells of the colonies.

![Fig. 8. Alpha-naphthyl acetate esterase activity in cells from monocyte-macrophage colony. (A) Weak activity in immature cells. (B) Intensive activity in mature cells.]
Acid Phosphatase

Both types of cells showed this enzymatic activity; it was weak in "young" cells and increased in intensity with their maturation. "Old" macrophages showed somewhat stronger enzymatic activity.

Peroxidase

Moderate activity was evident in eosinophils in the form of a few granules and diffuse cytoplasmic staining. No enzymatic activity was demonstrated in macrophages.

Peroxidase for Eosinophils

Positive staining was found only in mature eosinophilic granulocytes.

P.A.S. Staining

Moderate to strong positive reactions were found in cells of both types of colonies. Cells undergoing degeneration exhibited weakly positive reactions, if at all. In cells treated with ptyalin, P.A.S. staining was positive.

Toluidine Blue Stains

Almost every cell from the two types of colonies showed this metachromatic stain, when alcoholic toluidine blue was used. This staining is due to phagocytized agar particles composed of polysaccharides with SO₃H groups, which are responsible for pronounced, stable metachromasia.19 On the other hand, no metachromasia was found when aqueous solution of toluidine blue was used.

DISCUSSION

When human bone marrow cells were cultured in agar in presence of human spleen-conditioned medium, two types of colonies developed: diffuse and compact.

By means of employing a series of cytochemical methods as well as morphologic studies, we were able to provide more exact determination of characteristics of human bone marrow cells, grown in soft agar cultures.

Morphologic appearance and cytochemical properties indicate that the diffuse colonies consisted of monocyte-macrophages characterized by strong α-naphthyl acetate esterase activity, whereas the compact colonies were composed of eosinophilic granulocytes characterized by positive peroxidase, eosin, and Luxol fast blue staining.

Our observations of eosinophils developing in cultures suggest that a specific basic protein staining green with Luxol fast blue is produced in the cytoplasm of young eosinophils and later on concentrates into the specific granules.

By electron microscopy the specific granules may exhibit one of two structures: crystallloid core or a relatively pale internum.20 During the course of the present investigation, eosinophilic granules were found to be almost spherical with an electron-lucent center and an electron-dense periphery. In spite of the fact that the electron microscopic structure of eosinophilic granules in the cul-
tured cells is not identical with that known from human eosinophils from peripheral blood, the identical cytochemical properties nevertheless indicate that we are dealing with the same type of cytoplasmic structure which has been modified as a result of different developmental conditions.

The metachromatic staining testifies to the fact that these cells possess phagocytic activity. The cells undergo a process of degranulation with formation of phagosomes in which it was possible to identify the core of the granules. This suggests the possibility that in cultured eosinophilic cells as well, hydrolytic enzymes are localized in the matrix of the granules.

Using napthol AS-D chloroacetate as substrate, a strong esterase activity is considered to be characteristic of neutrophils. The fact that we found no marked AS-D chloroacetate esterase staining in the cultured cells is a further proof that these cells did not belong to the neutrophilic series. The low enzymatic activity found in some cells in both types of colonies may be due to phagocytosis of traces of neutrophils or to some changes affecting the cell membranes under conditions prevailing in the cultures.

With α-naphthyl acetate as substrate, we demonstrated strong enzymatic activity only in cells identified as macrophage-monocytes in large, diffuse colonies. Similar dark staining was found in monocytes of peripheral blood and bone marrow and reticulum cells, and therefore a common origin is likely.

No other types of colonies, such as neutrophilic granulocytes, were found, in spite of systematic and meticulous examination of all the colonies. This is in sharp contrast to the findings reported by others, which indicate the presence of neutrophilic colonies only, neutrophilic, mononuclear, and eosinophilic colonies, or granulocytic and mononuclear colonies.

These conflicting findings may be due to the lack of strict morphologic and cytochemical criteria for the definition of the colonies' cells. The differences in the methods of cultivation may also contribute to the discrepancies encountered.

The phenomenon that only these two types of cells, macrophages and eosinophils, were induced to differentiate and divide may be explained by the conditions prevailing in our in vitro system. Our results indicate that spleen-conditioned medium contains factors regulating monocytopoiesis and eosinopoiesis and that this activity requires the presence of F.C.S. It may be that in order to obtain proliferation of polymorphonuclear neutrophils, basophils, or other types of cells in vitro, different supplementary factors are required.

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