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Characterization of Monolayer and Organ Cultures of Cloned and Enriched Lymphohematopoietic Stromal Cell Populations

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The role of hematopoietic microenvironments in the regulation of maturation and differentiation of hematopoietic cells, although heavily debated, remains uncertain. Several investigators have suggested that the adherent "stromal" cell populations, which grow as colonies in cultures of lymphomyeloid tissues, include the cells involved in such regulatory processes. Grossly, the colonies described by several investigators appear similar morphologically, and the cells giving rise to them have been variously termed 1) fibroblast colony forming cells (FCFC), 2) plaque forming units-culture (PFU-C), 3) macrophage colonies, and 4) marrow stromal cells. FCFC have been reported to re-establish their parent microenvironment when transplanted in an allogeneic system. In this study, cloned and enriched cell populations obtained from such colonies in cultures of murine lymphomyeloid tissues have been characterized by their growth in culture and using morphological, histochemical, and electron microscopic techniques. The results demonstrated that, although the initial stromal colonies appeared to be identical, the constituent cell types varied considerably. Some colonies were comprised primarily of macrophages, while others appeared to contain predominantly fibroblasts; two additional cell types that established colonies have not yet been satisfactorily identified. These results demonstrate the heterogeneity of lymphomyeloid stromal colonies. There is a need for caution in the analysis of experiments in which uncharacterized stromal cell colonies are transplanted or employed as supporting monolayers in culture systems in experiments designed to evaluate the origins and functions of lymphohematopoietic stroma.

Key words: hematopoietic stroma, cloning, in vitro culture

Considerable evidence indicates that the proliferation and differentiation of hematopoietic cells is influenced differentially by the microenvironmental milieu provided these cells in various lymphohematopoietic tissues. Several investigators have suggested that the adherent "stromal" cell populations, which grow as colonies in cultures of these tissues, include cells involved in hematopoietic regulatory processes [1–6]. Friedenstein and

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associates have grown stromal colonies that they term fibroblastoid cells in liquid cultures from mouse, guinea pig, rabbit, and human hematopoietic tissues [1, 7]. The frequency of these colony-forming cells in mouse bone marrow was approximately 2 per 10^5 cells, and the size of the colonies ranged from 50–1,000 cells. When marrow-derived colonies were grafted under the kidney capsule in an allogeneic situation, ossification containing active marrow was observed at the graft site [8]. When spleen-derived colonies were grafted under the kidney capsule, aggregates of lymphoid cells developed. The results of these experiments were interpreted to indicate that the "fibroblastoid" colonies were in fact hematopoietic stromal colonies capable of supporting specific hematopoietic cell proliferation.

Metcalf [3] has postulated that the morphologically similar fibroblast-like colonies grown from marrow or spleen may be composed of totally different cells with different functions in lymphohematopoietic regulation. We have grown adherent colonies from hematopoietic stroma and characterized, in part, enriched and cloned cells from these colonies on the basis of their morphology and growth characteristics in vitro. The results presented below support the former portion of Metcalf's postulate that marrow stromal colonies appear to be composed of different cell types.

MATERIALS AND METHODS

Animals

C57B1/6 mice were purchased from Jackson Labs (Bar Harbor, Maine) or were obtained from our own breeding colony. All animals were in good health when utilized for these studies.

Enrichment by Physical Manipulation

Clusters of cells termed fibroblast colony forming cells (FCFC) and plaque forming units-culture (PFU-C) by Friedenstein et al [1] and Greenberg et al [6], respectively, were isolated by partial trypsinization and physical manipulation. Primary monolayers of mouse lymphohematopoietic tissues were subjected to trypsinization while under observation with an inverted phase contrast microscope. When the desired cluster of cells was noted to be loosening, a sterile 10 μl pipette was used to remove it from the flask. It was placed in a well of a microtiter plate, and cultured in Fischer's medium supplemented with 20% horse serum.

Enrichment by Differential Trypsinization

Primary monolayers were subjected to trypsinization and, every 2–5 min, the trypsin solution and any suspended cells were poured off into another culture flask containing Fischer's medium. Fresh trypsin was again added to the primary monolayer, and this process was continued for approximately 30 min, or until most of the cells in the primary monolayers were removed.

Cloning of Stromal Elements

Cloning of individual stromal cells was accomplished from cultures enriched in a manner described above. The limiting dilution technique of Cooper [9] was used for cloning individual cells. The isolated cells were cultured in Fischer's medium and observed daily with phase contrast microscopy.

Morphological Evaluation of Monolayers

During the culture period, monolayer development was regularly observed with an inverted phase contrast microscope. Representative culture flasks or glass coverslips within the flasks were periodically removed for histochemical and/or electron microscopic analysis. Specific stains used in this study for light microscopy included Wright's-Giemsa, toluidine blue, hematoxylin and eosin, periodic acid-Schiff, Masson's trichrome, Wilder's reticulum, methenamine silver and α-napthyl acetate esterase. Electron microscopy of the monolayers was accomplished using a modification of the technique described by Chang [10].

Organ Cultures

Cultures of stromal cells were trypsinized, centrifuged, washed, then resuspended in a small quantity of medium to make a thick cell slurry. Aliquots (5 μl) of this cell slurry were placed on sterile Nucleopore filters floating on medium in a Petri dish. The cells were incubated at 37°C in a humidified 5% CO_2 in air atmosphere. After 24–48 h, they were processed for electron microscopy.

RESULTS

Initial attempts at isolating individual colonies of stromal cells were made using spleen adherent cell (SAC) cultures. Fifty apparently identical colonies were removed by trypsinization and mechanical manipulation. Each colony removed was placed in a well of the microtiter plate. Of these 50 colonies, only two grew when incubated at 37°C. These two cultures were periodically passaged and expanded to 25 cm^2 culture flasks. The two populations appeared quite different when viewed with the phase contrast microscope (Fig. 1). Cultures of cells derived from spleen colony 1 (SC_1) were comprised of cells much smaller than those of spleen colony 2 (SC_2), and were pyramidal to dendritic in shape. SC_1 cells grew to confluence in 2–3 days after passaging and grew in a layer that was several cells thick. When viewed with the phase contrast microscope, large nuclei with multiple large nucleoli could be seen. In addition, dark granules and a few vacuoles were observed in the cytoplasm. One interesting nonmorphological observation was that within 30 seconds of adding the trypsin, most of the cells were removed from the substrate.

Cultures of cells derived from SC_2 were totally different than SC_1 cultures. The cells were large stellate cells with attenuated cytoplasm. Their morphology when viewed with a phase contrast microscope strongly resembled that of bone fragment-derived stromal cells (BFSC) [11, 12]. There were numerous cytoplasmic "ridges" stretching from the nuclear area to the periphery of the cell. The nuclei were large with two or more prominent nucleoli. A few granules were observed in the cytoplasm. After passaging, these cells grew to confluence in about 4 weeks and appeared to show contact inhibition of proliferation. These cells were quite resistant to trypsinization; only a few of the cells lifted off of the substrate even after 30 min of trypsinization and agitation.

Thirty apparently identical colonies derived from bone marrow adherent cell (BMAC) cultures were isolated by trypsinization and physical manipulation. Of these 30 colonies, only one grew when cultured in a well of the microtiter plate. When cell numbers had reached an acceptable level, the cloning procedure, described previously, was followed and 480 microtiter wells were prepared. Each well containing only one cell was marked, and of the marked wells, only one demonstrated cellular proliferation after

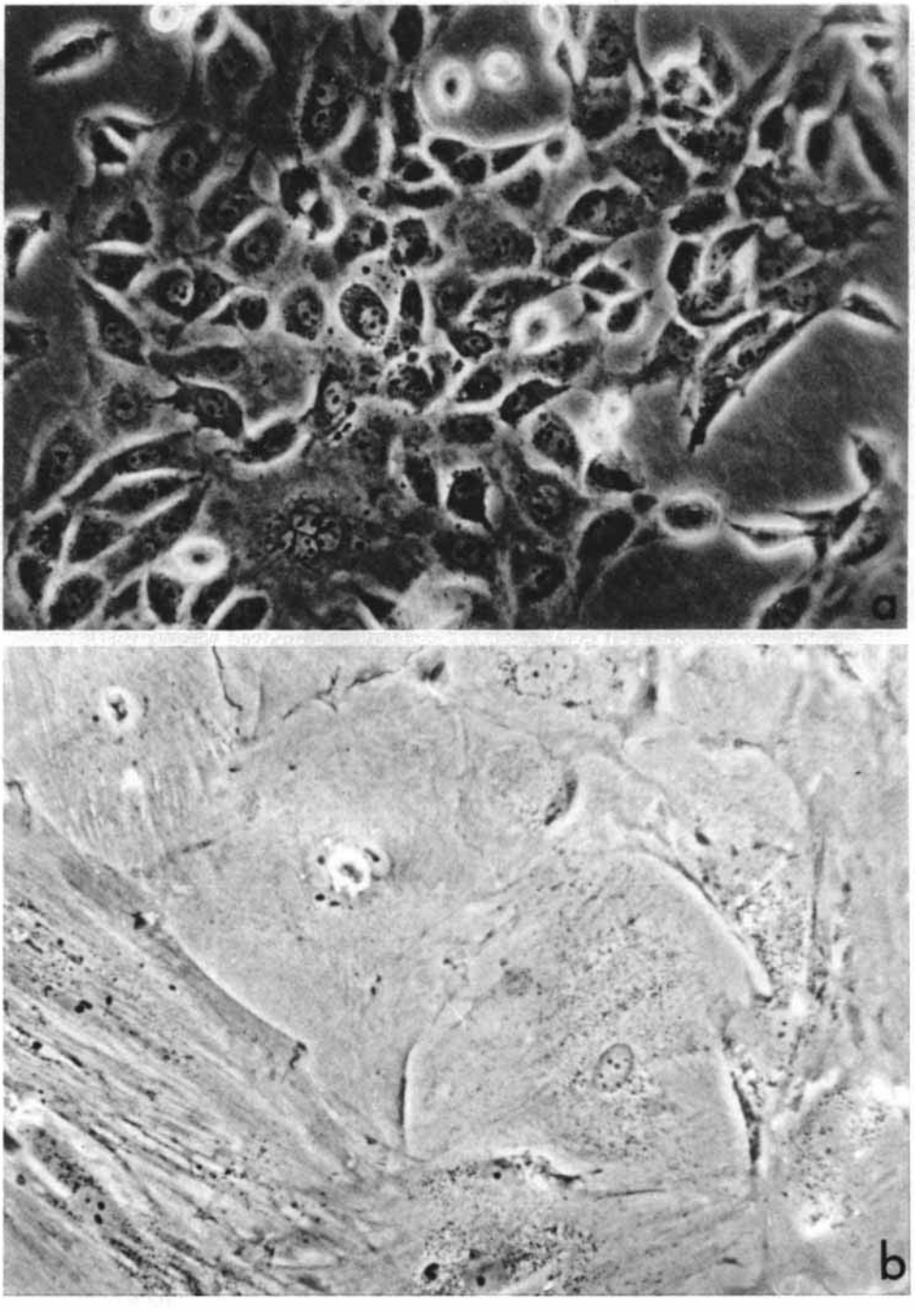

Fig. 1. Appearance under phase contrast microscopy of spleen-derived enriched, (ie, single colony derived), stromal cell cultures after several passages. a) SC_1 and b) SC_2. (× 320).

1½ weeks. Following numerous passages in progressively larger culture vessels, a confluent monolayer was formed in a 25 cm^2 "T" flask – 4 months after the original colony was isolated from the primary culture. These cells resembled the SC_2 cells and BFSC, as they were large stellate cells with attentuated cytoplasm (Table I). The nuclei were large with two or more prominent nucleoli. They grew very slowly and trypsinized off of the substrate only after a great deal of trypsin exposure and forceful agitation.

Marrow colonies (MC) 1, 2, and 3 were derived by isolating 65 apparently identical colonies from BMAC cultures. Their appearances under phase contrast microscopy are shown in Figure 2. MC_1 was fast growing and trypsinized readily. When viewed by phase contrast microscopy, they resembled SC_1 in cell size, shape, and nuclear morphology. MC_2 was slow growing, resisted trypsinization, and morphologically resembled SC_2. MC_3 was a rapidly growing cell type that took 3–4 min for removal with trypsin. The MC_3 cells were dendritic and slightly larger than those in MC_1 or SC_1. They had cytoplasmic processes that tended to stretch across surrounding cells and intertwine with processes of other cells. When viewed with phase contrast microscopy, they did not resemble any of the other enriched or cloned cell types. MC_4 and MC_5 were both derived from bone marrow cultures using the differential trypsinization technique. MC_4 resembled the fast-growing pyramidal cells such as SC_1 and MC_1. MC_5 on the other hand, consisted of large slow-growing stellate cells such as those found in MC_2 and SC_2. These slow growing cells either died out or were overgrown by other faster growing cells by the third or fourth passage. These results are summarized in Table I.

Cells from MC_1, MC_2, and MC_3 were passaged into T flasks containing glass coverslips. Following a culture period adequate to allow growth of cells on the coverslips, they were removed and placed in a 10% buffered formalin solution. A cell line (T131) derived from cultured murine thymus supplied by Drs. D.A. Crouse and R.K. Jordan was prepared in the same manner. The coverslips on which cells had been growing were stained with the histochemical stains described above. The morphology of the cells growing on the coverslips was readily demonstrated after staining them with hematoxylin and eosin. The MC_1 cells, which resembled T131 cells when viewed with the phase contrast microscope, were a heterogeneous population comprised of pyramidal as well as dendritic

TABLE I. List of Cell Populations Isolated From Spleen and Marrow, the Method of Isolation, and Some General Characteristics of the Populations

	Derived from	Isolation technique[a]	Growth	Trypsinization rate	Relative cell size	Cell shape	Number of passages[b]
SC_1	Spleen	T & PM	Fast	Fast	Small	Pyramidal and dendritic	8
SC_2	Spleen	T & PM	Slow	Slow	Large	Stellate	4
MC_1	Bone marrow	T & PM	Fast	Fast	Small	Pyramidal	40+
MC_2	Bone marrow	T & PM	Slow	Slow	Large	Stellate	4
MC_3	Bone marrow	T & PM	Fast	Intermediate	Medium	Dendritic	20+
MC_4	Bone marrow	DT	Fast	Fast	Small	Pyramidal	14+
MC_5	Bone marrow	DT	Slow	Slow	Large	Stellate	3
Marrow clone	Bone marrow	T & PM – Clone	Slow	Slow	Large	Stellate	7

[a]T & PM = trypsinization and physical manipulation. DT = differential trypsinization.
[b]+ = line still going.

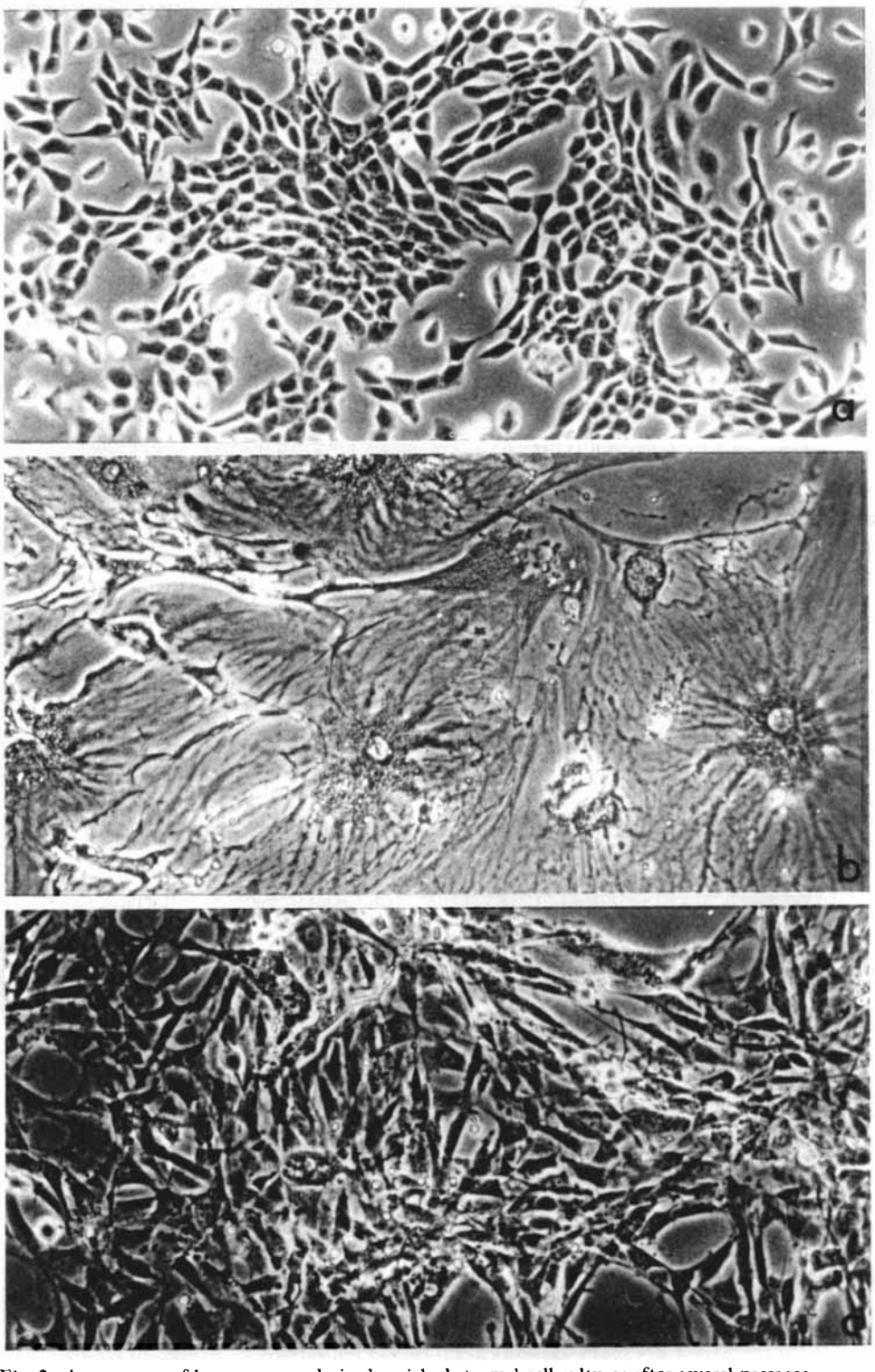

Fig. 2. Appearance of bone marrow-derived enriched stromal cell cultures after several passages. a) MC_1, phase contrast; b) MC_2, Wright's-Giemsa; and c) MC_3, phase contrast. (× 100.)

cell types. They resembled MC_3 cells more closely than T131. The MC_1 cells were PAS-positive, and Masson's trichrome stain demonstrated many ribosomes in the cytoplasm as well as a few extracellular aggregates of matrix material. Wilder's reticulum stain revealed no collagen surrounding the cells; however, methenamine silver did demonstrate a potential collagen precursor within the cytoplasm of some of the cells. The possibility exists that this staining was due to the presence of polysaccharide and not the presence of collagen or its precursors.

MC_2 cells were large stellate-shaped cells with extensive cytoplasm. They were PAS-positive, and ribosomes were demonstrated in the cytoplasm by Masson's trichrome stain. Extensive black deposits were observed in the cytoplasm of these cells following staining with methenamine silver. MC_3 cells were dendritic shaped and tended to grow with cytoplasmic processes crossing over and under surrounding cells. The cells were PAS-positive, and no evidence of collagen or its precursors was demonstrated. The cells of the T131 thymus-derived cell line were also PAS-positive, although to a lesser degree than the other cell types. The cells were pyramidal in shape and Masson's trichrome stain demonstrated numerous ribosomes in the cytoplasm. No collagen was demonstrated with any stain.

Attempts to define the lineage of the marrow colony derived cells using histochemical stains were not entirely successful in that not all the cells could be fully identified. As is summarized in Table II, all three of the marrow-derived populations were PAS-positive, and their cytoplasm contained many ribosomes. Masson's trichrome stain revealed some extracellular collagenous material in MC_1 cell cultures, but there was none observed in MC_2 or MC_3. Some potential collagen precursors were demonstrated in the cytoplasm of most of the MC_2 cells by staining with methenamine silver. Some of the MC_1 cells also demonstrated the characteristic black precipitate. T131 cells resembled MC_1 and MC_3 in their staining characteristics. At the phase contrast level, they also strongly resembled SC_1 cells. The cultures were analyzed for macrophage contamination by examining their abilities to phagocytize opsinized yeast and by staining them with α-napthyl acetate. With the exception of MC_3, the cultures were composed of less than 1% macrophages. The numbers of macrophages in MC_3 declined with each passage.

Electron microscopy was accomplished on SC_1 and SC_2 cells, which resembled MC_1 and MC_2, respectively, at the light microscopic level. Examination of the micrographs demonstrated conclusively that SC_1 cells and SC_2 cells derived from apparently identical colonies in spleen cultures were indeed different cell types. SC_1 cells were smaller than

TABLE II. Summary of the Results of the Histochemical Stains Utilized in This Study on One Thymic-Derived and Three Marrow-Derived Cell Populations

	H & E	PAS	Masson's trichrome	Wilder's reticulum	Gomori's methenamine silver	α-Napthyl acetate (%)
MC_1	Dendritic and pyramidal shape	+	Collagen+ Ribsomes+	Collagen−	Collagen+,−	0.9
MC_2	Stellate shape	+	Collagen− Ribosomes+	Collagen−	Collagen+	0.2
MC_3	Dendritic shape	+	Collagen− Ribosomes+	Collagen−	Collagen−	< 50
T131	Pyramidal shape	+	Collagen− Ribosomes−	Collagen−	Collagen−	0

SC_2 and contained prominent nucleoli. Extensive networks of rough endoplasmic reticulum were characteristically dilated in the SC_1 cells. Golgi apparatus, numerous mitochondria, and large quantities of glycogen were commonly seen in the cytoplasm (Fig. 3a). SC_2 cells were notable for their large size and the absence of nucleoli in the nuclei, which displayed perinuclear heterochromatin. The most characteristic feature of the cytoplasm was the presence of bundles of tonofilaments projecting across the entire cell. A few mitochondria and glycogen granules were also observed in the cytoplasm (Fig. 3b).

The organ cultures of the various cell populations demonstrated that the appearance of the aggregated cells was characteristic for each population, ie, grossly, MC_1 and MC_4 organ cultures were flattened and yellowish, while MC_3 organ cultures were white and rounded. Thick sections (1μ) of the araldite-embedded organ cultures demonstrated further differences between the populations of cells. The MC_1 and MC_4 cells were pleomorphic and appeared to be in quite random arrangements (Fig. 4a). On the other hand, MC_3 cells were pleomorphic but appeared to be organized in parallel concentric arrays. Many cells at the center of the MC_3 organ cultures exhibited pyknotic nuclei and cytoplasmic degeneration (Fig. 4b).

Electron microscopy of these cells grown as organ cultures showed that MC_1 cells contained irregular nuclei with peripheral heterochromatin and prominent nucleoli. The cytoplasm contained an abundance of glycogen, a few lipid vacuoles, and rough endoplasmic reticulum. Several junctional complexes were observed between cells (Fig. 5). Cells of the MC_3 cultures contained more rounded nuclei with peripheral heterochromatin and nucleoli. Extensive rough endoplasmic reticulum and many lipid vacuoles were characteristic of the cytoplasm. Between cells, many junctional complexes could be seen (Fig. 6). No extracellular collagen was observed in organ cultures of any of these cell populations. No hematopoietic cells could be identified in these cultures.

DISCUSSION

These results demonstrate that, although lymphohematopoietic stromal cell colonies appeared to be morphologically identical at the gross level, the constituent cell types varied considerably in their characteristics, ie, from a single culture, colonies composed of totally different cell populations were isolated. Furthermore, colonies comprised of identical cell types were present in cultures of different lymphomyeloid tissues, ie, bone marrow, spleen, and thymus. Consequently, it is dangerous to conclude in studies of transplantation of aggregates of individual colonies that a particular stromal cell type is responsible for transferring a given hematopoietic microenvironment. The results presented here demonstrate that individual colonies contain several distinct cell types which might interact to provide a suitable microenvironment for hematopoiesis or lymphocyte differentiation. When aggregates of colonies are transplanted many different cell types are transferred, and it is unreasonable to conclude that any one cell type is responsible for establishing the new microenvironment. These reservations apply to transplants performed in a syngeneic situation. There are additional reservations concerning the original studies of Friedenstein [8], which were performed in an allogeneic situation. These studies, in our hands, have proved to be difficult to repeat in the syngeneic system.

At the present time, we cannot identify with certainty all the cellular constituents of the colonies that we have grown. It is apparent that macrophages and fibroblasts are two of the constituent populations. Other populations of cells may be subpopulations of fibroblast-like cells, eg, preadipocytes, or they may belong to other cell lineages such as

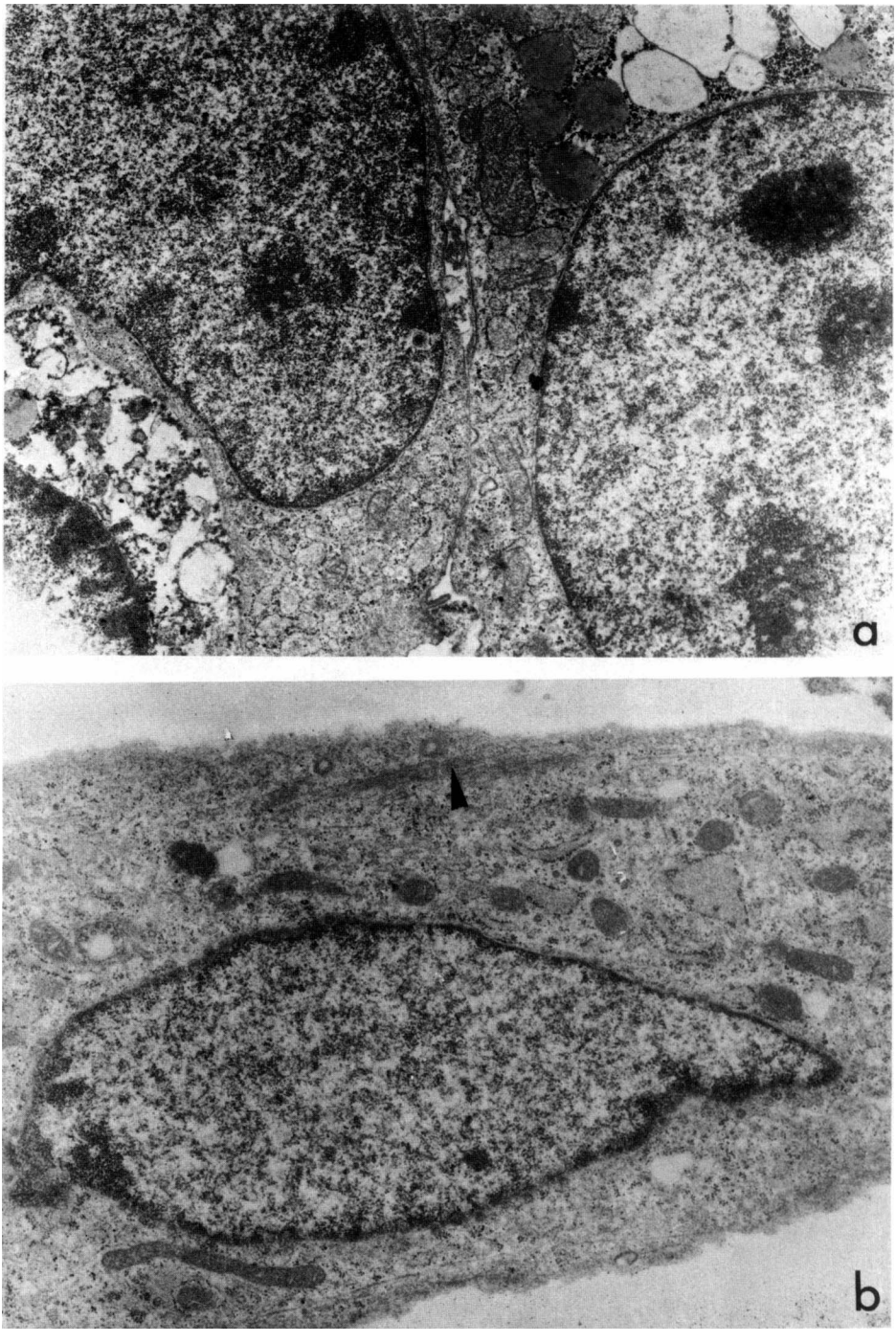

Fig. 3. a) Electron micrograph of SC_1 monolayer. Note the multiple nucleoli, numerous cytoplasmic organelles and the absence of collagen. (× 12,000.) b) Electron micrograph of SC_2 monolayer. Note the absence of nucleoli, perinuclear heterochromatin, and bundles of tonofilaments (arrow). (× 14,000.)

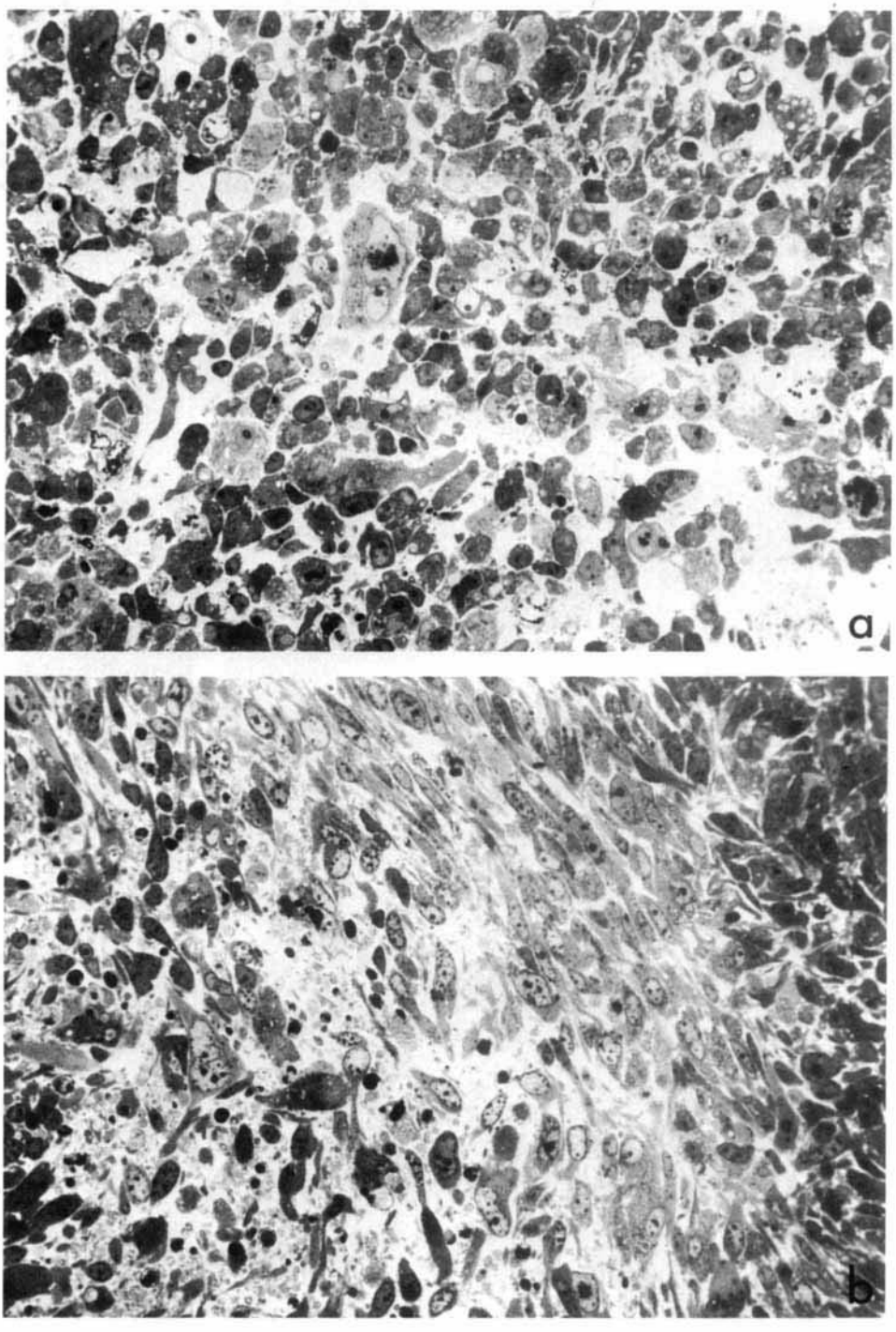

Fig. 4. a) Section of MC_1 cells following 24 h of organ culture (toluidine blue). (× 200.) b) Section of MC_3 cells following 24 hr of organ culture (toluidine blue). (× 200.)

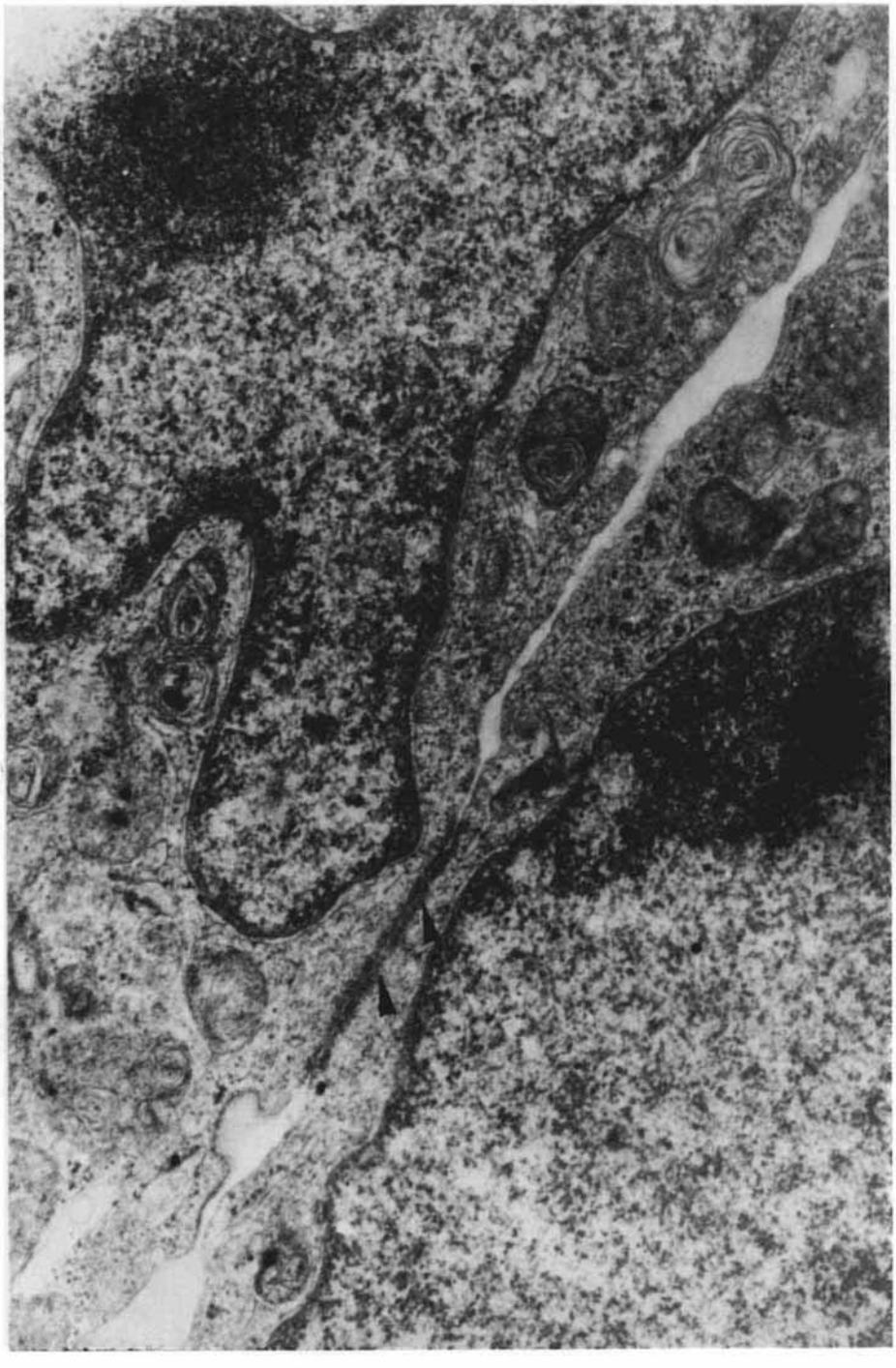

Fig. 5. Electron micrograph of MC_1, stromal cell organ culture. Note nucleoli, gap junction (arrows), and the absence of collagen. (× 42,000.)

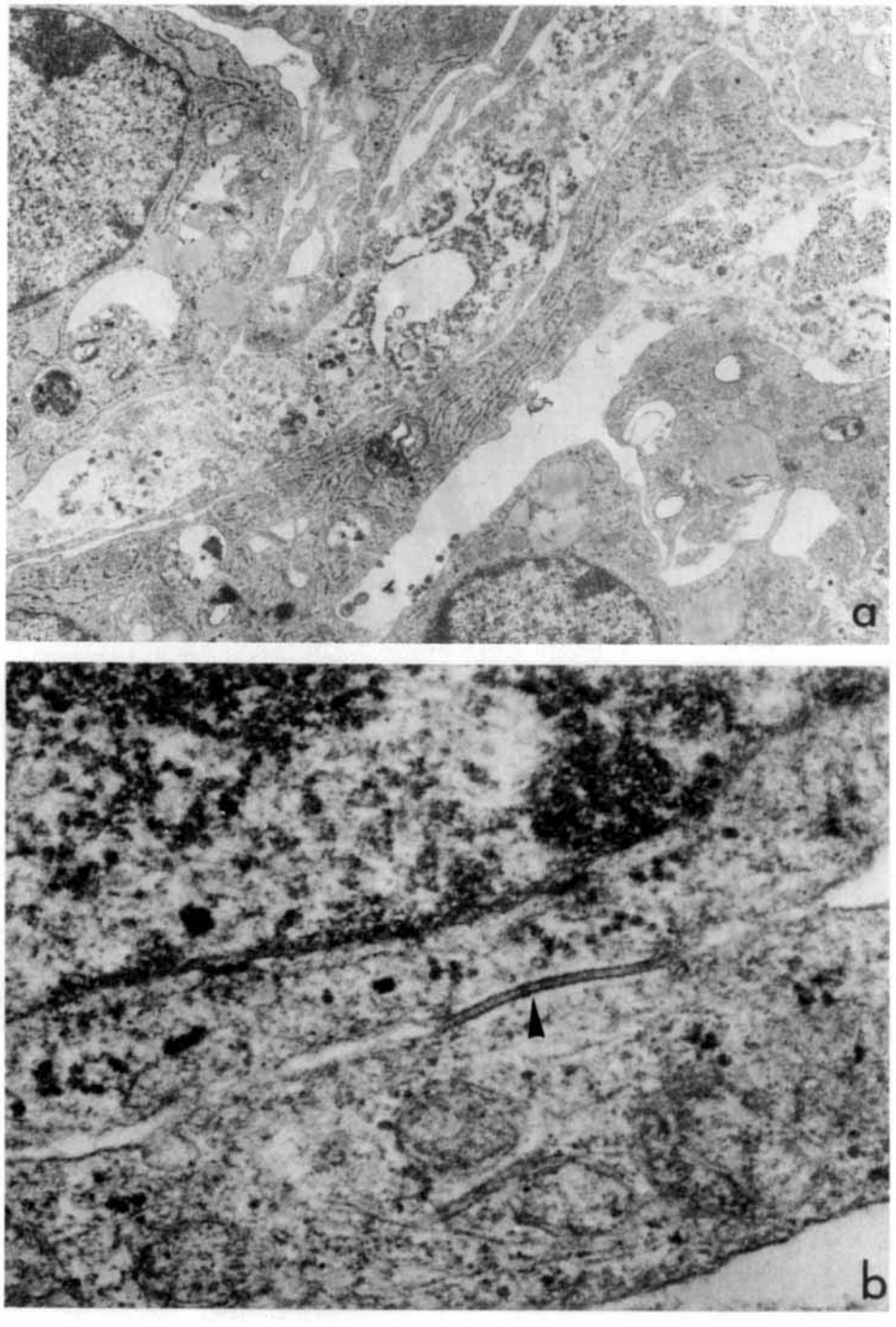

Fig. 6. a) Electron micrograph of MC_3 stromal cell organ culture. Note nucleoli, extensive rough endoplasmic reticulum, and lipid vacuoles. (× 13,000.) b) Representative gap junction (arrow) in organ culture of MC_3 stromal cells. (× 84,000.)

endothelium (T131?) or osteoprogenitor cells (BFSC?). There are many morphological and a few functional attributes that can be listed to aid in this identification; however, none of these characteristics provides for definitive identification. It is evident that analysis for specific cell markers, such as prekeratin, factor VIII, and platelet adherance, will have to be performed to identify definitively these various cell types. A potentially useful observation concerning the cloning of these cells was that in the situation of selective trypsinization of established monolayers followed by washing to remove trypsin, the growth rate of the surviving colonies (clones?) was much faster than the growth rate of the cells that originally established the monolayer. Gospodarowicz [13] has described the use of pre-prepared (basement membrane and glycosaminoglycans coated) flasks for the growth of anchorage-dependent cells. Additionally, a technique has been reported [14] by which the culture substrate is prepared with a basement membrane fiber component derived from the same cell types of those to be cultured. Differentiated cell populations have been shown to function more readily in such cultures. In our system, the use of trypsin rather than a detergent to remove the original monolayer cells would have reduced the quality of the conditioning cell material on the surface of the flasks. Even so, hematopoietic stromal cells still grow better on such partially conditioned flasks than on unprepared plastic surfaces.

Numerous technical problems were encountered during the cloning of the lymphohematopoietic stromal cells. Cells that are cloned or grown in low numbers do not grow readily, and it is felt that the application of the technique of precoating the flasks would increase the numbers of successfully cloned cells. Furthermore, the estimates of cloning efficiency and growth rates presented here, ie, 1 in 20–30 stromal colonies contains cells with a high clonogenic potential, may be in part or wholly artifactual in that they may reflect the ability of the different cell types to "auto-condition" their growth environment. The use of precoated flasks might considerably increase the estimated numbers of clonogenic cells and lead to revised estimates of the growth rates and adherence properties of the different cell types found in lymphohematopoietic stromal colonies.

Finally, the functions of these various cells in the regulation of hematopoiesis have yet to be determined. Studies are currently underway that should demonstrate any abilities of these cells to produce or bind hematopoietic regulatory factors and to maintain CFU-S in vitro or to reproduce the hematopoietic microenvironment following engraftment.

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