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METHODS FOR EVALUATING THE MORPHOLOGICAL AND IMMUNOHISTOCHEMICAL PROPERTIES OF HUMAN TUMOR COLONIES GROWN IN SOFT AGAR

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SUMMARY

Clonogenic assays have been widely adopted for the investigation of hematopoietic and human tumor stem cell biology. Inasmuch as specific, whole colonies need to be analyzed morphologically, we used various methods for fixing and embedding individual colonies in situ that allowed macroscopic, light microscopic (LM), immunofluorescence, and transmission electron microscopic (TEM) evaluation of the intact colony. Melanoma colonies stained with Masson's Trichrome, hematoxylin and eosin (H&E), periodic acid-Schiff, Best's carmine, Page-Green method for inclusion bodies, and Snook's reticulum revealed cellular and extracellular components by LM. Ultrastructural studies revealed specific cellular organelles and extracellular components. Immunofluorescence studies demonstrated cell-surface fibronectin, a high molecular weight, adhesive glycoprotein. Myeloma colonies contained a heterogeneous cell population and produced amyloid fibers that were observed by TEM. Fixation and embedding the colonies in agar for TEM has several advantages over centrifugation methods and other conventional techniques for collecting cells in that (a) an entire specific colony can be studied, (b) there is excellent preservation of the cell and its spatial orientation in the colony, and (c) the extracellular matrix (ECM) of the colony is preserved for immunohistochemical analysis.

Key words: melanoma; myeloma; clonogenic assay; fibronectin; extracellular matrix; immunofluorescence.

INTRODUCTION

The growth of cells in semisolid medium has been used extensively to investigate properties of hematopoietic and solid tumor clonogenic cells (1,2). Cells from human malignant melanomas (3,4), multiple myelomas (5), and other tumors (2) have been grown in semisolid medium. Several properties of human tumor colonies have been investigated, including chemotherapeutic sensitivity (6,7), karyology (4,8), self-renewal capacity (9,10), and light microscopic characteristics (3,4,11). However, detailed morphological analysis has been difficult for several reasons: (a) multicellular colonies are spherical or ellipsoidal in shape so that simple air drying of the agar bilayer distorts the spatial orientation of the tumor cells (11), (b) centrifugation techniques flatten the colony such that the cells' position and relationship in the colony are lost (12), and (c) removal of the colonies by aspiration or scooping with a pipette collects extraneous cells in addition to the cells of the colony (13).

We have applied morphological and immunohistochemical methods to study intact isolated colonies that differentiate as distinct morphological variants from the same tumor material. The fixation and embedding procedure preserves the colony shape and has allowed detailed LM, TEM, and immunofluorescence analyses. We used this method to investigate the cellular composition of human malignant melanoma and multiple myeloma colonies and to contribute to the

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histological and immunological characterization of intact colonies. Two variants that can be distinguished by pigmentary features have been demonstrated for melanoma colonies. Heterogeneity in cell size, pigmentation, shape of colony, and presence or absence of material surrounding the cells were noted. The production of amyloid fibers from cells in myeloma colonies has also been demonstrated.

Materials and Methods

Human melanoma clonogenic cell assay. Our culture conditions for clonogenic melanoma cells were a minor modification of the bilayer agar assay developed by Hamburger and Salmon (14) and have been described extensively (4). Briefly, single cell suspensions were obtained from 26 biopsies of subcutaneous nodules from patients with metastatic malignant melanoma in accord with a protocol approved by the University of Arizona Committee on Human Subjects. Tumor tissue was cut into 1 mm^2 pieces, placed into a 50 ml conical tube, and inverted several times. Tumor pieces and macroscopic clumps were allowed to settle at unit gravity, and the supernatant fluid containing the single cells was aspirated. The cells were then cultured in 30 × 10 mm dishes (Falcon) with a 1.0 ml of underlayer containing 0.5% agar (Bacto agar, Difco, Detroit, MI), Ham's F10 medium, 10% heat inactivated fetal bovine serum (FBS), penicillin (100 μg/ml), and streptomycin (100 U/μl). The plating layer was the same medium in 0.3% agar with freshly added animal derived insulin (1.54 U/ml), glutamine (0.45 μg/ml), pyruvate (0.34 μg/ml), and mercaptoethanol (0.77 m*M*). One-half million cells were plated per dish. The dishes were incubated in a 5% CO_2:95% air atmosphere at 37° C with constant high humidity. Several hundred colonies were prepared for analyses between 8 and 50 d in culture.

Human myeloma clonogenic cell assay. The culture system has previously been described in detail (5,7). Briefly, bone marrow cells were obtained from patients by sternal or iliac puncture after informed consent was obtained. Cells were aspirated into a heparinized syringe, mixed in an equal volume of 3% dextran-saline, and sedimented at room temperature for 45 min. The cells in the supernatant fluid were collected after centrifugation at 150 ×*g* for 10 min and washed twice in Hanks' balanced salt solution with 10% heat-inactivated FBS. The cells were then suspended in 0.3% Bacto agar in CMRL 1066 medium supplemented with 15% horse serum, penicillin (100 U/ml), streptomycin (100 μg/ml), glutamine (2 m*M*), vitamin C (0.3 m*M*), insulin (3 U/ml), asparagine (0.6 mg/ml), and DEAE dextran (0.5 mg/ml) to yield a final concentration in the range of 5×10^5 cells/ml. A freshly prepared dilution of 2-mercaptoethanol (Sigma Chemical Co., St. Louis, MO) was added to give a final concentration of 5×10^{-5} *M* immediately before plating of the cells. One milliliter of the resultant mixture was pipetted into a 35 mm plastic petri dish containing 0.25 ml of oil primed BALB/c mouse spleen conditioned medium (2) in a 1 ml feeder layer of 0.5% Bacto agar. Cultures were incubated in a 5% CO_2:95% air atmosphere at 37° C that was humidified.

Culture plates were examined carefully by inverted microscopy after initial plating to ensure that no aggregates were present and sequentially during the culture period to evaluate ongoing colony growth. Ten colonies were analyzed after 19 d in culture.

Colony selection. Colonies were selected under an inverted light microscope and identified by making a circle on the petri dish directly below the colony. A characteristic pattern of circles for each petri dish was made so that a specific colony could be traced from the macroscopic level to the TEM level.

Light microscopy of melanoma colonies. The agar plates were generally fixed by gently immersing the entire plates for 4 h or longer at room temperature (25° C) in 10% neutral buffered formalin. Histochemical stains requiring a special fixative, such as Bouin's solution for glycogen, were used as needed. Fixation was followed by routine paraffin embedding of the entire agar plate in an Autotechnicon (Model 2A, The Technicon Company, Chauncey, New York). The agar was lightly stained occasionally with a few drops of toluidine blue before being transferred to a 70% ethanol bath for the purpose of visualizing the normally transparent agar. Thick serial sections (6 to 7 μm) of the entire bilayer agar were readily obtained by keeping the agar bilayer flat from the time of fixation to final embedding in paraffin and then cutting sections in a cold room at 4° C. Approximately 400 serial sections of the agar were cut, placed on cardboard trays, and then stored at room temperature. Every 10th section was floated onto Chrom-Alum slides and stained with H&E to locate colonies in the agar. Selected sections were then stained with Masson's Trichrome, H&E,

periodic acid-Schiff, Best's carmine, Page-Green method for inclusion bodies, and Snook's reticulum.

Immunofluorescence studies of melanoma colonies. The expression of cell-surface fibronectin by human melanoma cells in colonies was determined by the indirect immunofluorescence technique as follows. Agar plates containing melanoma colonies were fixed overnight at 4° C in absolute ethanol and glacial acetic acid (99:1) and processed according to the methods of Sainte-Marie (15). After dehydration the tissues were embedded without infiltration in paraffin (56° C). The entire embedding process lasted less than 5 s, and paraffin blocks were immediately placed on a cold plate after the paraffin was poured, thus preventing subsequent protein denaturation. The entire agar bilayer was sectioned serially (6 to 7 μm) in a cold room, and ribbons of sections were placed onto cardboard trays and stored at 4° C. Every 10th section was stained with H&E to locate the colonies in agar. Selected sections known to contain colonies were then mounted on Chrom-Alum slides in a waterbath (40° C). After deparaffinization at 4° C, all slides were rinsed overnight in 0.15 *M* Tris buffer, pH 7.4.

Rabbit antisera and absorbed antisera (control) to sodium dodecyl sulfate gel purified large external transformation sensitive protein was a gift from Dr. Richard O. Hynes, M.I.T., Cambridge, MA. The specificity and biochemical characterization of this antisera has been described previously (16). The primary antisera and absorbed antisera were applied to the sections [1:20/phosphate buffered saline (PBS)] overnight at 4° C in a humidified chamber. After three 2-min rinses in PBS, the secondary antibody (rhodamine conjugated goat antirabbit IgG, Cappel Labs, Inc., Downington, PA) was applied to sections in a dilution of 1:30/PBS for 1 h at room temperature in the dark. After this incubation, the slides were rinsed three times for 2 min in PBS, mounted with PBS and glycerol (10:90) and cover slips, and viewed in a Zeiss fluorescence standard-18 microscope. All photomicrographs were taken with Ilford HP-5 film at a standard exposure time.

Electron microscopy. Agar plates containing either melanoma or myeloma colonies were fixed by gently layering on several milliliters of modified Karnovsky fixative (17) (1% paraformaldehyde; 1.25% glutaraldehyde; pH 7.4; 560 mOsM) and allowing the plates to stand at room temperature for 90 min. The agar plates were then rinsed in 0.2 *M* cacodylate buffer and postfixed in 2% OsO_4 buffered in 0.072 *N* cacodylate buffer, at pH 7.4 for 1 h. The plates were washed in 0.144 *N* cacodylate buffer, dehydrated in increasing concentrations of ethanol, followed by 100% propylene oxide, and embedded overnight in a 50:50 mixture of propylene oxide/Spurr. Colonies in the agar plates previously selected before fixation were re-identified under the dissecting microscope, and small blocks of agar containing one or two colonies were cut out carefully with a scalpel and placed in 100% Spurr for 24 h. The small blocks were transferred to fresh 100% Spurr for final embedding. Alternatively, the entire agar bilayer of the 50:50 mixture of propylene oxide/Spurr was placed in 100% Spurr for 24 h, and then transferred to fresh 100% Spurr in an embedding pan. Specific colonies were reidentified by matching the entire agar mold to the individual pattern of the corresponding petri dish. Thick and thin sections were cut with glass knives on a Porter-Blum MT2 ultramicrotome. Thick sections were stained with toluidine blue. Thin sections were mounted on uncoated 200 mesh copper grids and stained with uranyl acetate and lead citrate (18,19). Grids were examined in a Philips 300 transmission electron microscope using 60 kV accelerating voltage.

Results and Discussion

Two variants of melanoma colonies are identified, a hypomelanotic and a hypermelanotic type. Figure 1 illustrates a hypomelanotic melanoma colony 490 μm in diam as observed in soft agar with inverted light microscopy. This colony had been in culture for 50 d and is classified as the large light variant, as described by Meyskens and Salmon (3). The size of the cells that comprise the periphery of the colony may be measured directly without difficulty. The size of the cells in the center of this ellipsoid colony cannot be determined due to resolution problems associated with cell stacking. Additionally, Fig. 1 illustrates the absence of a demonstrable accumulation of melanosomes around the periphery of the colony. The hypermelanotic variant (Fig. 2) generally forms smaller colonies than the hypomelanotic variant. Individual cells of the two variants are approximately the same size. Small dark structures characteristically form a halo at the periphery of the hypermelanotic colony (Fig. 2), and by TEM evaluation are known to contain single and aggregated melanosomes (20).

Serial sections of paraffin embedded colonies allow three dimensional evaluation and utilization of various stains on similar sections. Sections prepared for routine H&E staining demonstrate melanoma cells surrounded by an extracellular matrix containing fibrillarlike material embedded

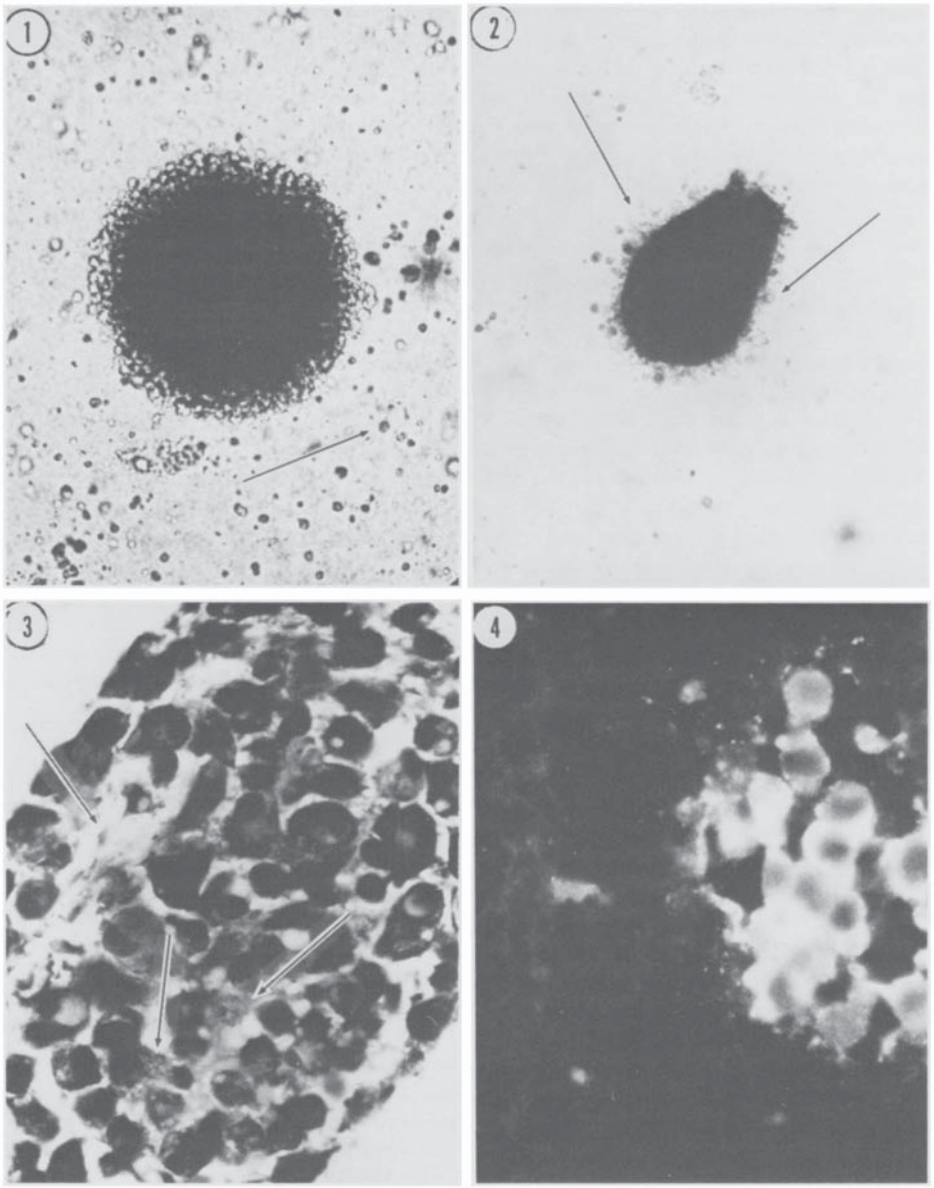

FIG. 1. A hypomelanotic human melanoma colony 490 μm in diam, as observed after 50 d in soft agar. Nonclonogenic cells from the single cell preparation are present throughout the agar layer and can be seen surrounding the colony (*arrow*). ×74.

FIG. 2. A hypermelanotic human melanoma colony cultured for 8 d is 185 μm in diam. A dark coloration and a halo of melanosomes (*arrows*) are characteristic for this variant. ×150.

FIG. 3. Connective tissue (*arrows*), specifically stained by the Masson's trichrome method, is entirely localized within the colony. It is an acellular connective tissue stroma and occupies much of the intercellular space within the colony. This hypomelanotic colony was grown for 25 d in soft agar. ×475.

FIG. 4. Immunohistochemical staining for fibronectin indicates a positive reaction of the melanoma cell membrane and of the cell-surface associated extracellular matrix. The colony was maintained in culture for 28 d. ×320.

within a homogeneous matrix. Snook's reticulum stain demonstrates reticulum fibers throughout the extracellular matrix of the entire colony. Masson's trichrome histochemical procedure demonstrates a positively stained connective tissue (fibrillar and homogeneous) in the extracellular space of a hypomelanotic (large light) colony grown for 25 d (Fig. 3). Transmission electron microscopy has provided the necessary resolution to identify the positively stained connective tissue material as wispy, nonstriated fibrils (Figs. 5 and 6).

Additional histochemical stains used in this study were periodic acid-Schiff and Best's carmine, which failed to show a demonstrable amount of glycogen within the cells. Also, inclusion bodies were not observed with the Page-Green method.

For immunohistochemical analysis, preservation of antigenicity is a serious consideration. For this reason, the agar plates were fixed and processed at a minimum temperature. A great deal of care was taken in selecting the proper tissue preparation for the preservation of the fibronectin antigen. The overnight rinse in 0.15 *M* tris buffer before antibody labeling should have had no harmful effects on the antigen inasmuch as the antigen was fixed well. The dehydrant fixative (absolute ethanol and glacial acetic acid) was used in this study because it is considered to be one of the most popular fixatives for immunocytochemistry inasmuch as the loss of antigenic reactivity is minimal (21). In general, antigens located within the cell can be stained if the cell membrane has been permeabilized by acetone, ethanol, a detergent, or a combination of these agents. The solvents used in the Sainte-Marie (15) technique, in conjunction with sectioning, should have permeabilized the cell membrane sufficiently to allow the antigen-antibody reaction to occur intracellularly, on the cell membrane, and extracellularly. Fibronectin staining associated with the cell membrane and adjoining ECM was noted in all observed colonies and is demonstrated in Fig. 4. Although the most optimal resolution is not available with immunofluorescence microscopy on 6 to 7 μm thick sections, current studies in progress reveal similar results of fibronectin staining specifically on the cell membrane and associated ECM using the peroxidase-antiperoxidase (PAP) technique on 1 μm thick sections of melanotic colonies. Fibronectin, a high molecular weight glycoprotein, has been shown to be involved in cell attachment to a substratum (22) and cell-to-cell contact (23). The presence of fibronectin can be used to distinguish carcinomas from sarcomas in vivo (24). Our data show the presence of this glycoprotein associated with cell surfaces of human melanoma cells within a forming colony. These results are in contrast to the loss of fibronectin during transformation which has been reported in several systems [for review *see* (25)]. This contrast is to be expected inasmuch as fibronectin production varies from system to system and from in vivo to in vitro observations (25). It is interesting to note that immunohistochemical techniques were applied to cryostat sections that were lightly fixed in a buffered aldehyde solution. After using this method, the result indicated that the cytological preservation was poor, and it was difficult locating colonies embedded in the frozen cryoprotectant media.

The ultrastructural detail of cells from the hypomelanotic variant is shown in Figs. 5 and 6. In general, the nuclei are large, have irregular borders, and contain prominent nucleoli. The stages of melanosome maturation are similar to those described in vivo. However, the parallel tubular arrays found in metastatic melanomas and in tissue culture by other investigators (26) were not observed by us. Therefore, the presence of premelanosomes and melanosomes are the ultrastructural criteria for diagnosing melanomas in our laboratory. Cell junctions are found in neither the hypomelanotic nor the hypermelanotic colonies grown in soft agar. This observation is in agreement with Mackay and Osborne (26) who have noted either the absence of cell junctions in melanoma tumors in vivo or poorly developed cell junctions such as plaques.

Myeloma colonies are comprised of heterogeneous cells (Figs. 7 and 8). In Fig. 7, a 1 μm thick, toluidine blue stained section of a myeloma colony depicts plasma cells among myelocytes. Confirmation of the nature of the plasma cell is shown at the TEM level (Fig. 8) in which the major characteristic—the rough endoplasmic reticulum—is abundantly represented. Cell heterogeneity was also noted in a patient whose cells produced amyloid fibers and secreted them into the soft agar (Fig. 9). This amyloid deposition was observed in the majority of colonies from this patient. The ultrastructural data obtained from the clonogenic assay for this amyloid pathology supplemented in vivo observations and have been reported in detail elsewhere (27).

Investigators have found it difficult to examine the morphology of cells grown in soft agar because

of the difficulties associated with preserving cytological architecture. Most of the reported techniques have analyzed hematopoietic colonies by LM (28), TEM (28,29), and cytochemistry

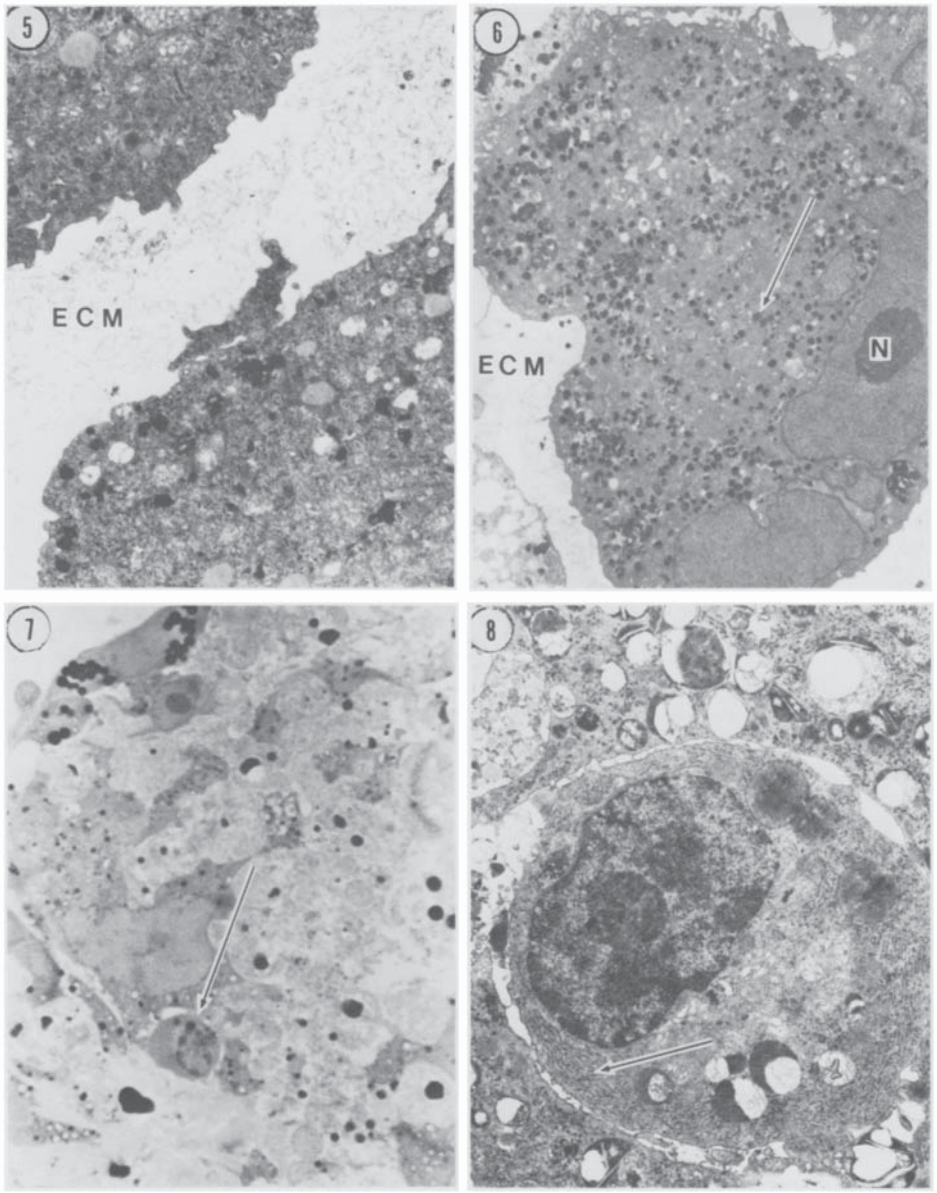

FIG. 5. Transmission electron microscopy illustrates the fibrillar ECM surrounding the human melanoma cells grown for 28 d in soft agar. ×6,500.

FIG. 6. Melanosomes (*arrow*) occupy the entire cytoplasm of this individual cell cultured in soft agar for 50 d. Nucleoli (*N*) are very prominent in the nuclei. ×3,300.

FIG. 7. The cellular heterogeneity of a myeloma colony cultured for 9 d is seen in this 1 μm thick, toluidine blue stained section. Several plasma cells are obvious. The cell identified by the *arrow* is visualized with TEM in Fig. 8. ×1,200.

FIG. 8. This plasma cell is encircled by myelocytes. The ultrastructural morphology is similar to that shown for plasma cells in vivo. Note the characteristic abundance of rough endoplasmic reticulum (*arrow*). ×6,900.

FIG. 9. Amyloid fibers (*arrows*) are produced by macrophagelike cells in the myeloma colony and released into the soft agar. The cells were in culture for 19 d. ×32,000.

(30,31). Recently Harris and coworkers (32) have reported on ultrastructural analysis of colonies grown in the bilayer agar system. Their TEM data on melanoma colonies correspond to our hypomelanotic variant and also confirm the correlation of melanosomes as ultrastructural markers for in vitro to in vivo comparisons. Although these studies and others have provided techniques to evaluate cellular morphology, our study extends these investigations by combining histochemical and immunohistochemical techniques with the ultrastructural analysis of human melanoma colonies grown in soft agar.

Our investigation provides information for studying an entire specific colony while maintaining each cell's spatial orientation, and for examining the extracellular matrix of the colony by LM, immunohistochemistry, and TEM. We are now extending our investigations to identify the components of the extracellular matrix produced by melanoma colonies grown in the soft agar bilayer system specifically in an attempt to elucidate the role of the extracellular environment in abnormal cellular differentiation.

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