Journal of Supramolecular Structure 11:517–528 (1979)
Tumor Cell Surfaces and Malignancy 359–370

Selection of Malignant Melanoma Variant Cell Lines for Ovary Colonization

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Murine melanoma line B16-F1, which shows some specificity for metastatic organ colonization of lung but rarely metastasizes to ovary, was used to select variant cell lines with increased preference for experimental ovary metastasis. Ovary-colonizing melanoma cell lines were sequentially selected in syngeneic C57BL/6 mice by repeated intravenous administration and surgical recovery of ovarian melanoma tumors for tissue culture. After ten selections for experimental ovary metastasis, line B16-010 was established which formed experimental metastatic ovary tumors in almost every test animal. In tissue culture B16-010 cells grew in circular colonies with rounded, smooth cell peripheries compared to B16-F1 cells which were flatter, grew in irregular patterns, and exhibited long cellular projections. Ovary-selected B16 lines contained less melanin pigment (B16-010 $<$ B16-05 $<$ B16-01 $\cong$ B16-F1) compared to the parental melanoma line. Together with previous cloning and selection data, these results are consistent with the preexistence of highly malignant cells in the parental tumor population that possess the ability to metastasize to specific organs.

Key words: cell variants, electron microscopy, malignant melanoma, melanin, metastasis

Tumor cells are characteristically uncontrolled because of their abilities to proliferate in an unregulated fashion, circumvent normal differentiation processes, and thwart normal cellular interactions that maintain proper cell positioning and movement [1, 2]. In malignant neoplasms these properties lead to invasion of surrounding normal tissues and dissemination to form new tumor colonies (metastases) at near and distant host sites [3–5]. Distant metastatic colonization occurs when invading malignant cells penetrate and are released into the lymphatics, coelmoic cavities, or circulating system. However, many of these transported malignant cells die and only a small fraction are thought to survive to form new tumor colonies [6–8].

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Received May 31, 1979; accepted July 16, 1979.

0091-7419/79/1104-0517$02.30

Metastasis via blood-borne dissemination does not always result in tumor colonization of tissues and organs based strictly on circulatory tracks. In many experimental animal models of metastatic tumor spread, the locations of gross metastases are nonrandom and do not parallel malignant cell distribution or the initial capillary beds encountered [9–18], suggesting that factors other than nonspecific trapping in the microcirculation are involved in tumor cell arrest. After their initial arrest malignant cells may die, invade and grow, or detach and recirculate to colonize other sites [12, 13, 19, 20]. During circulatory transport a variety of cellular interactions take place such as homotypic adhesion of tumor cells to form multicell emboli [21–23], heterotypic adhesion of tumor cells to platelets [24], lymphocytes [25, 26], and noncirculating host cells [23, 27], and these may affect subsequent malignant cell arrest and survival.

In order to study tumor cell and host properties important in metastatic tumor spread, animal tumor models have been developed that show reproducible metastatic behavior in syngeneic hosts and where low or nonmetastatic tumor lines are available for direct comparison [5, 28]. Several of these models have been based on Fidler's sequential selection procedures [29] to obtain metastatic variant lines of B16 melanoma with altered preference for lung [25, 29] or brain [14–16] tumor colonization. Here we describe in vivo selection of B16 melanoma variants that show increased preference for experimental ovary metastasis.

MATERIALS AND METHODS

Cells

The murine melanoma line (B16-F1, selected once for lung colonization [29], was obtained from Dr. I. J. Fidler (National Cancer Institute–Frederick Cancer Research Center, Frederick, Maryland). This cell line, which shows some specificity for lung but also forms experimental metastases at a variety of extrapulmonary sites [12–14], was used for subsequent in vivo selection of melanoma cell lines that preferentially colonize brain [14–16] or ovary. Cells from tumor minces were grown in tissue culture dishes or flasks (Corning Plastics) in Dulbecco-modified Eagle's medium (DMEM) supplemented with 10% fetal bovine serum, nonessential amino acids, and antibiotics (either 50 μg/ml) gentamicin or 100 units/ml penicillin plus 100 μg/ml streptomycin). Cell cultures which had just attained confluency were used for biologic assays, usually between the second and tenth passage in vitro.

Biologic Assays

Cells were detached from tissue culture surfaces with 2 mM ethylenediaminetetraacetic acid (EDTA) in Ca^{2+}-free, Mg^{2+}-free phosphate-buffered saline and suspended in serum-free DMEM. Viable cells (2.5×10^4) were injected in a volume of 0.2 ml into tail veins of ten female C57BL/6 mice, and the animals were sacrificed after 3–4 weeks. Lungs were injected via the trachea with 10% buffered formalin for fixation prior to scoring for lung melanoma colonies; all other organs were removed and examined under a dissecting microscope and the body cavities were examined for presence of gross tumor colonies.

Histology

Organs of animals previously injected with parental or ovary-selected B16 melanoma lines were surgically removed and fixed in 10% buffered formalin for 3–4 weeks. Organs were routinely processed for paraffin embedding and sectioning (5–10 μm), and mounted sections were stained with hematoxylin-eosin and examined.

Phase Contrast and Scanning Electron Microscopy

B16 malanoma cell lines were plated at various densities in T-25 tissue culture flasks (Corning Plastics) and grown for 3 days in DMEM plus 10% fetal calf serum. Sparse and confluent cultures were photographed with phase contrast optics (Nikon) at 100X.

For scanning electron microscopy B16 lines grown on glass cover slips were fixed in 1.5% glutaraldehyde in phosphate buffered saline, pH 7.2, for 10 min at 37°C and then 1–3 h at 22°C. The glutaraldehyde-fixed monolayers were postfixed in 1% osmium tetroxide in 1 mM $CaCl_2$–0.1 M sodium phosphate buffer, pH 7.2 for 1 h at 22°C. Monolayers were dehydrated through a graded series of ethanol, transferred to Freon 113 and critical-point-dried. After coating with 50–100 Å gold-palladium, the samples were observed in a Hitachi model S500 scanning electron microscope.

RESULTS

Selections, Biologic Assays, and Histology

Ovary-colonizing B16 melanoma variant lines were sequentially selected from B16-F1 by intravenous (tail vein) injection of single-cell suspensions of line F1 (100,000 cells per inoculum). Four weeks later rare ovary tumors were surgically removed and adapted to tissue culture to form line B16-01, which was further selected five and ten times to obtain lines B16-05 and B16-010, respectively. The number of cells per inoculum was gradually decreased with the latter sequential selections; the last injections for ovary selection employed approximately 20,000 cells. Assays for organ colonization were conducted by intravenous (tail vein) injection of 25,000 viable melanoma cells followed by examination after 4 weeks. In this assay B16-F1 yields an average of approximately 40–50 lung tumors per animal, but often fails to form ovary tumors (Table I). Line B16-01 shows similar biologic behavior to B16-F1; however, lines B16-05 and B16-010 form experimental ovary metastases in almost all animals injected and dramatically fewer lung tumor colonies (Table I).

Histologic examination of ovary tumors was performed on animals injected with the various melanoma lines. The rare ovary tumors formed in mice injected with lines

TABLE I. Experimental Metastasis After Intravenous Injection of B16 Melanoma Variant Lines

	Experimental metastases[a]		
Cell line	Ovary tumors	Average no. lung tumors (range)	Other tumors
B16-F1	0/10	47 (22–200)	2/10 thoracic 1/10 liver 1/10 adrenal
B16-01	0/10	53 (1–256)	3/10 thoracic
B16-05	8/10	32 (30–155)	2/10 thoracic 1/10 liver
B16-010	8/9	7 (1–17)	2/10 liver 1/10 adrenal

[a]25,000 viable B16 melanoma cells were inoculated intravenously into groups of ten animals and experimental metastases were determined after 4 weeks.

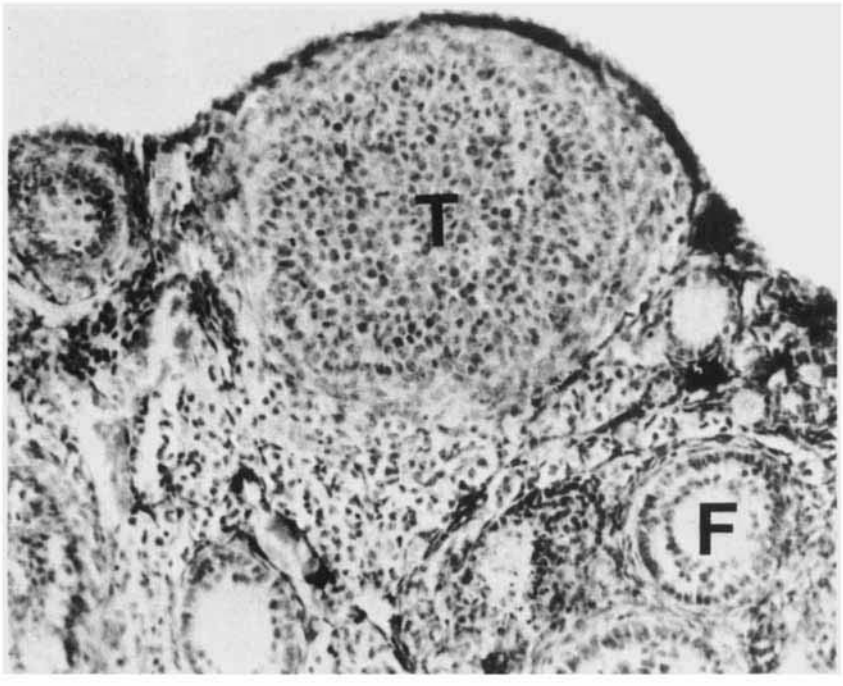

Fig. 1. Histology of ovary from B16-010-injected animal. T, tumor; F, follicle. × 248.

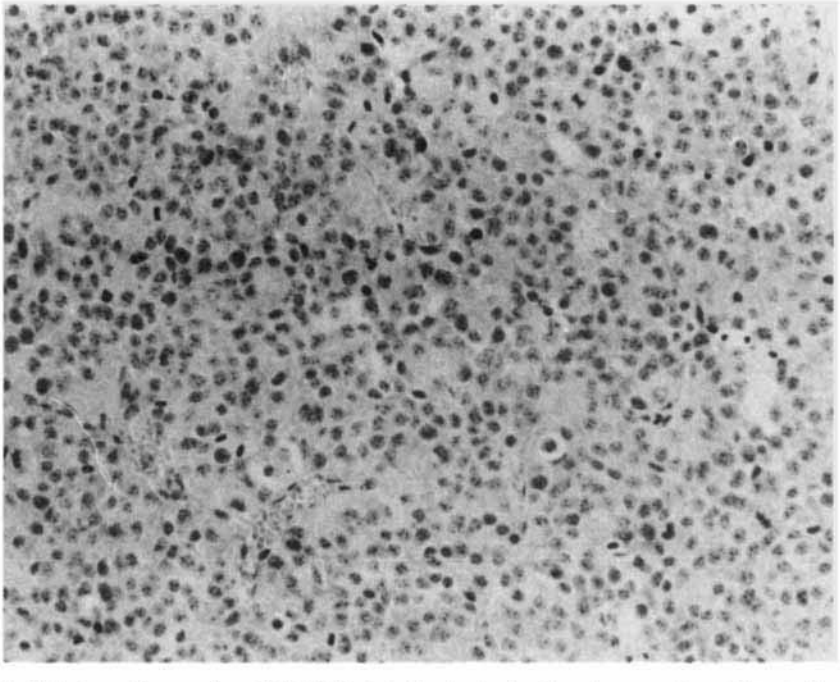

Fig. 2. Histology of ovary from B16-010-injected animal. Amelanotic tumor has obliterated ovary. × 248.

B16-F1 or B16-01 were pigmented and easily identified in ovary sections (Fig. 1). Ovaries from animals injected with B16–010 cells were greatly enlarged owing to the presence of amelanotic tumors, and examination of these ovaries revealed that the 010 tumors had obliterated the normal ovary structure (Fig. 2).

Cell Morphology and Pigmentation

Colony and cell morphology was assessed in vitro by phase contrast and scanning electron microscopy. Sparse B16-F1 cells were irregularly spaced and shaped with long

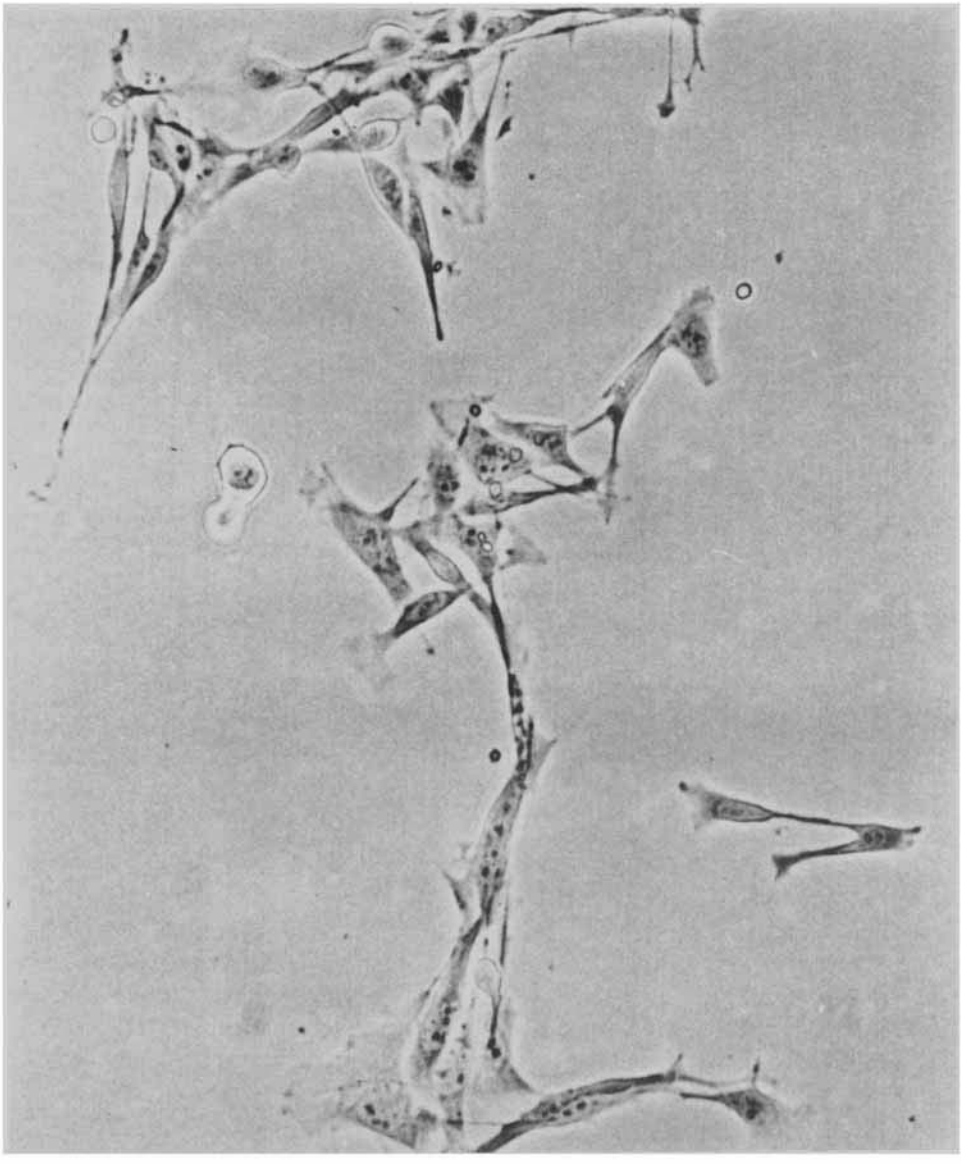

Fig. 3. Phase contrast micrograph of sparse B16-F1 cells in culture. × 300.

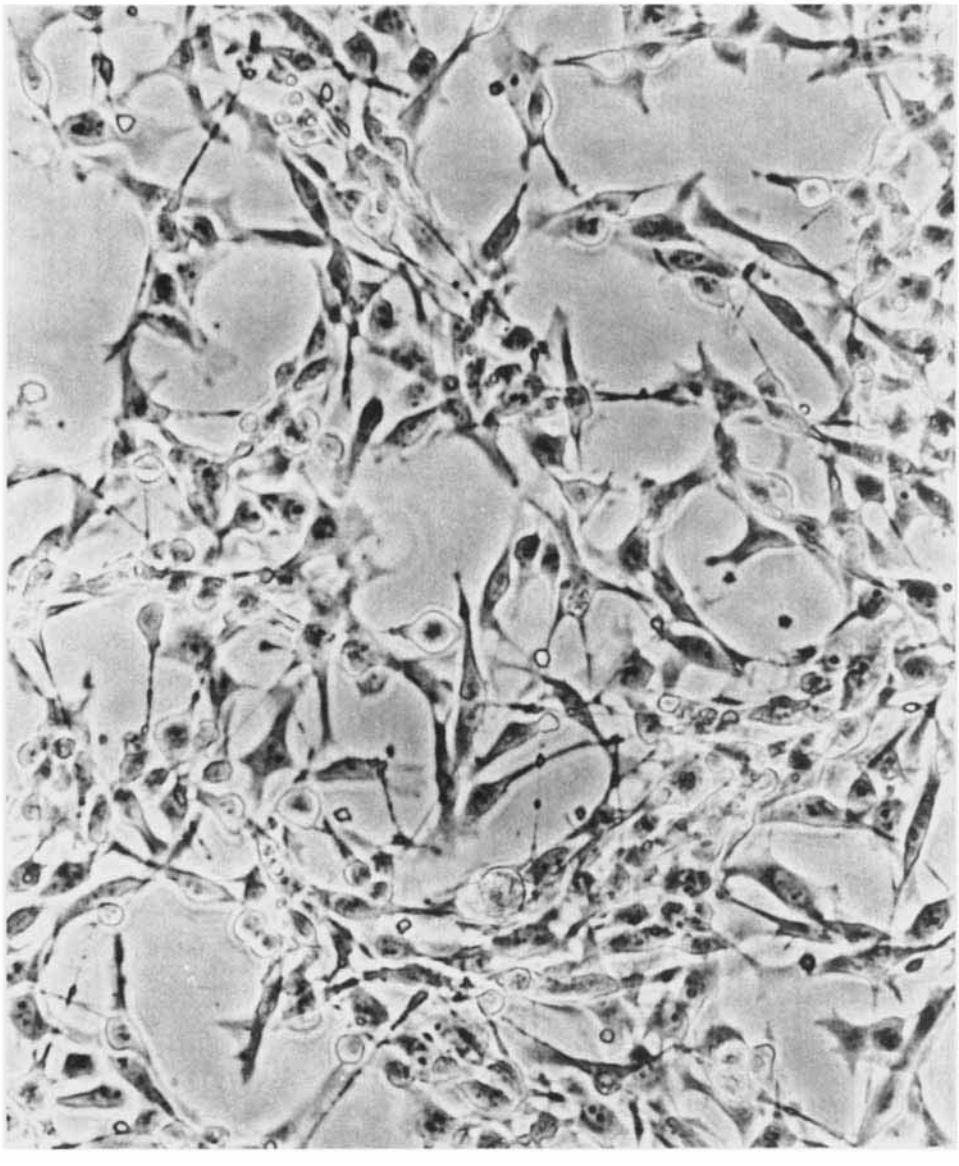

Fig. 4. Phase contrast micrograph of confluent B16-F1 cells in culture. × 300.

cytoplasmic extensions which often under- and overlapped adjacent cells (Fig. 3). At confluency this same pattern of irregular cell placement and multilayering was apparent (Fig. 4). Sparse B16-010 cells were flatter with fewer cytoplasmic extensions and grew in round-shaped colonies (Fig. 5). When B16-010 cells grew to confluency, this same pattern of growth persisted, and the cells seemed to form multilayers less readily than B16-F1 cells (Fig. 6). When examined by scanning electron microscopy, these morphologic differences were even more apparent. B16-F1 cells appeared very irregularly shaped at confluency, with numerous long cell projections and microvilli (Fig. 7), while B16-010 cells were rounder and contained fewer pseudopodia (Fig. 8).

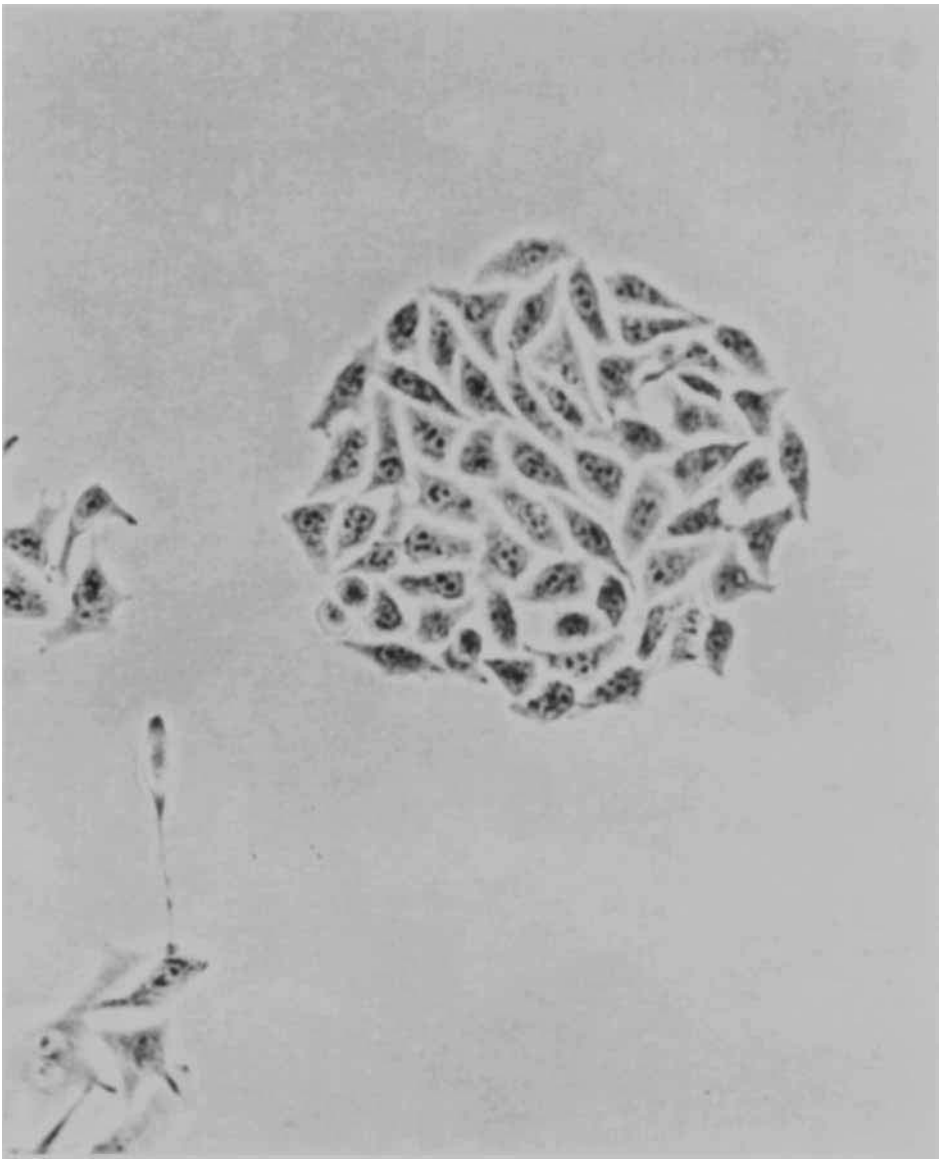

Fig. 5. Phase contrast micrograph of sparse B16-010 cells in culture. × 300.

The relative pigmentation in various B16 melanoma lines was examined by comparing cell pellets for the black pigment melanin. When line B16-F1 was compared to brain- and ovary-selected variant lines, only the latter selections showed dramatic differences (Fig. 9). Lines B16-05 and B16-010 exhibited a progressive loss of pigmentation with selection for ovary preference. That this is related to the number of in vivo selections was unlikely, since brain-selected lines B16-B5 and B16-B10N were similar in pigmentation to B16-F1 (Fig. 9).

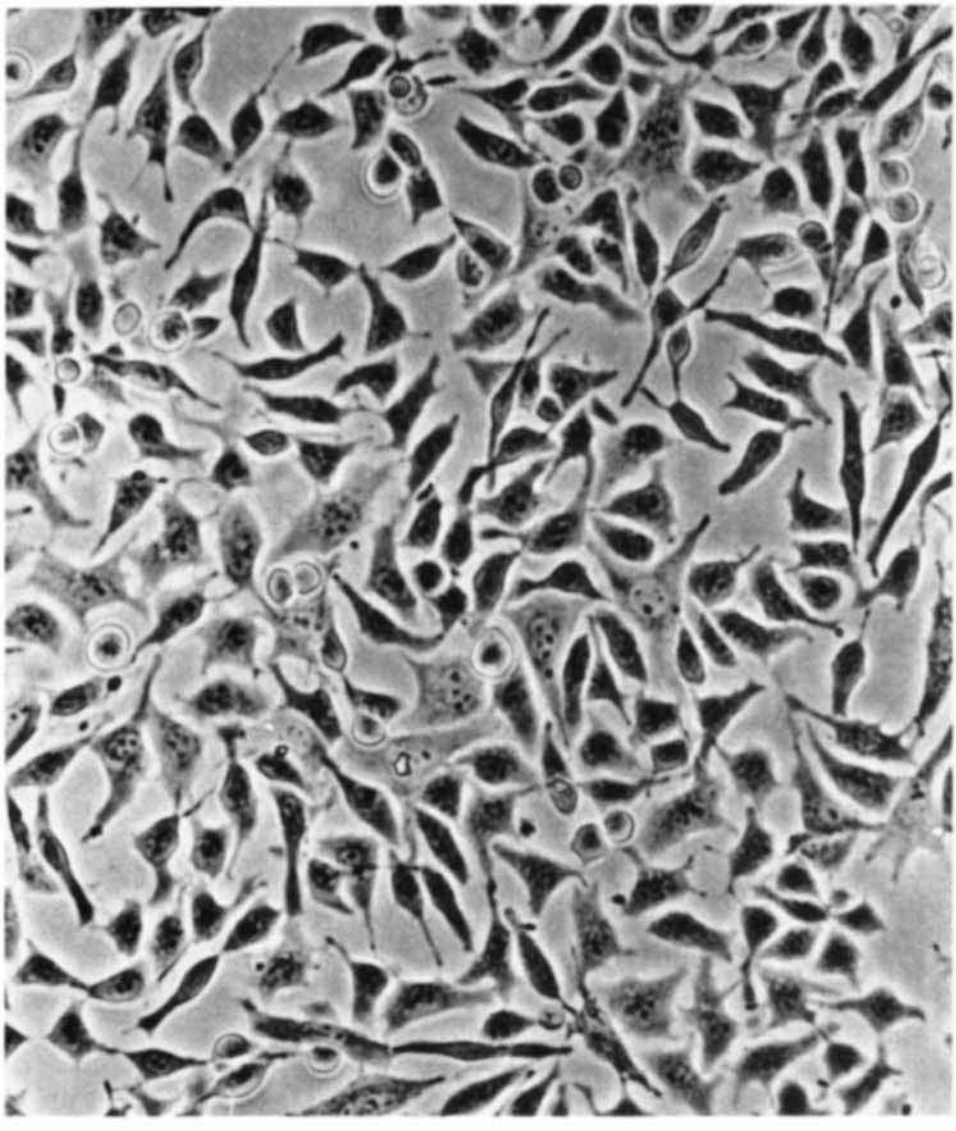

Fig. 6. Phase contrast micrograph of confluent B16-010 cells in culture. × 300.

DISCUSSION

Sequential selection for ovary-preferring B16 melanoma lines yielded increasingly more ovary-specific lines. Although line B16-010 was not totally specific for ovary and gave some experimental metastases at other sites, we have recently obtained a clone of B16-010 that appears to be quite specific in colonizing ovaries (K. W. Brunson and G. L. Nicolson, unpublished). In the biologic assays utilized here tumor cells were intravenously injected via the tail vein so that they had to pass through pulmonary

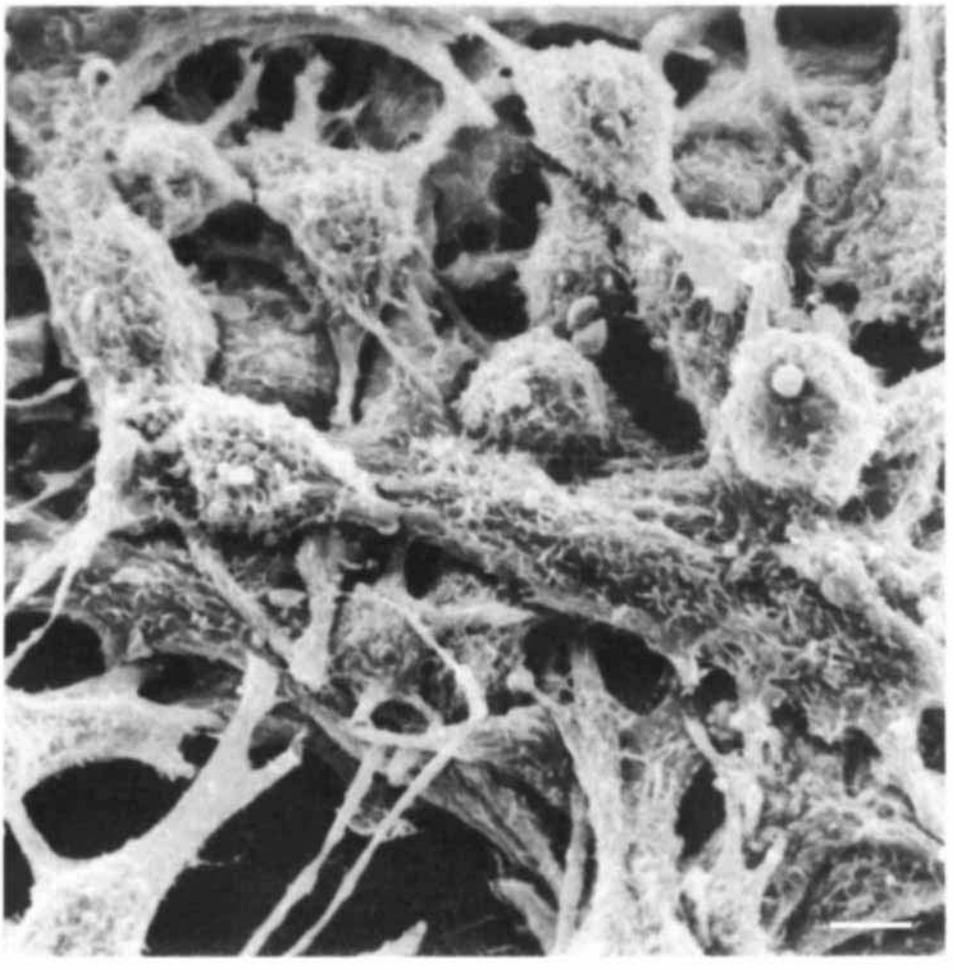

Fig. 7. Scanning electron micrograph of B16-F1 cells at confluency. Bar equals 5 μm.

capillaries and recirculate to reach the ovaries. Similar assays for brain-selected B16 lines have also shown that variant cell lines can be selected that are capable of recirculating from sites of initial arrest and finding their way to target organ sites [15, 16].

The in vivo and in vitro properties of ovary-selected lines suggest that they may represent a subpopulation of the parental B16-F1 melanoma cells. Cell and colony morphology in vitro are distinctly different from B16-F1 in the ovary-selected series, and there is a marked difference in melanin pigmentation. Preliminary cell surface labeling studies using lactoperoxidase-catalyzed ^{125}I-iodination techniques indicate that lines B16-05 and B16-010 have two exposed surface proteins that differ in amounts or exposures compared to parental line B16-F1. When analyzed by sodium dodecyl sulfate-polyacrylamide electrophoresis autoradiography, lines B16-05 and B16-010 show increased ^{125}I-incorporation in surface proteins of approximately 140,000 and 150,000 mol wt, which correlates with the number of in vivo selections and preference for ovary

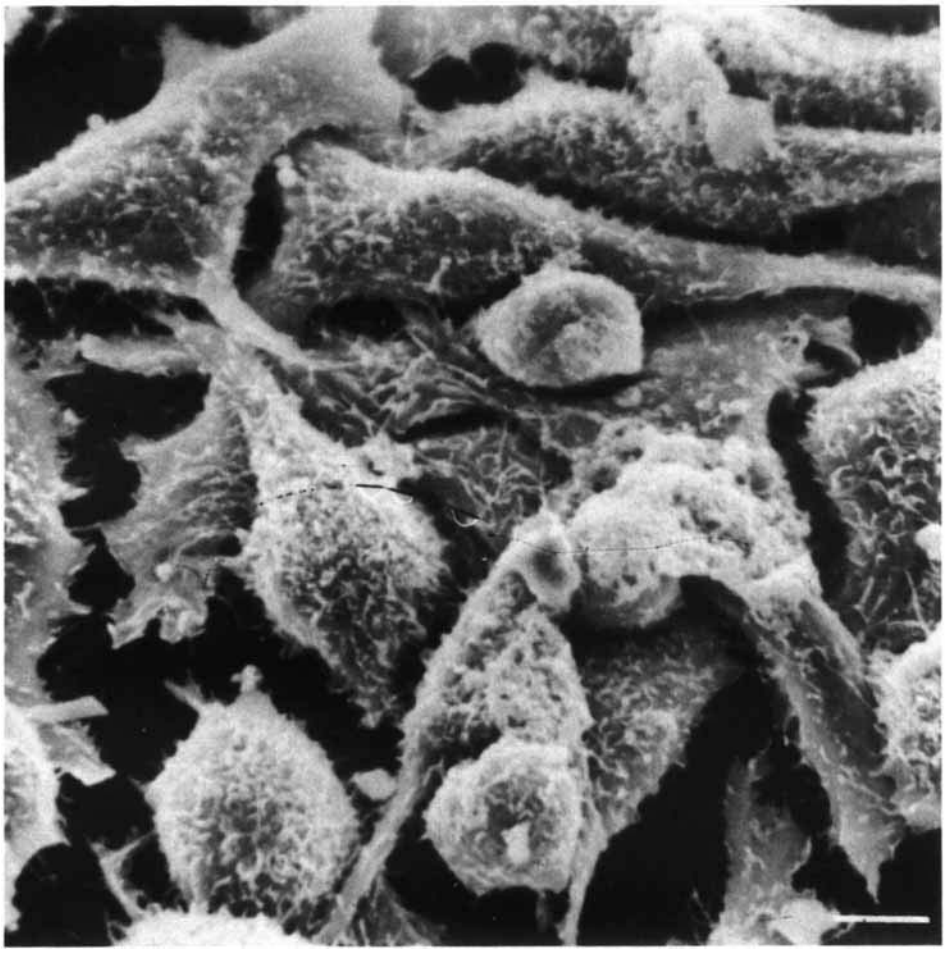

Fig. 8. Scanning electron micrograph of B16-010 cells at confluency. Bar equals 5 μm.

metastasis. In contrast, variants selected for brain colonization show different changes which correlate with their in vivo properties. Lines B16-B5 and B16-B10N show increased exposure to lactoperoxidase-iodination in proteins of approximately 95,000 and 100,000 mol wt which correlates with the number of in vivo selections and preference for brain metastasis [15]. Therefore, certain identifiable surface proteins are different in each of the selected series, and these changes may be important in directing implantation at specific organ sites in vivo [27].

The successful selection of metastatic variants with altered organ preference suggests that the original parental line B16-F1 and perhaps its precursor may be heterogeneous with respect to its phenotypic properties. Either these highly metastatic variant cells preexisted in the primary tumor population, or the phenotypic variants could have arisen by a process of organ adaptation during the selection process. Two different types of experiments have been performed to answer this question. First, primary tumor cell populations have been cloned and fluctuation tests carried out to determine whether highly metastatic variant cells preexist in the parental population. Experiments with B16

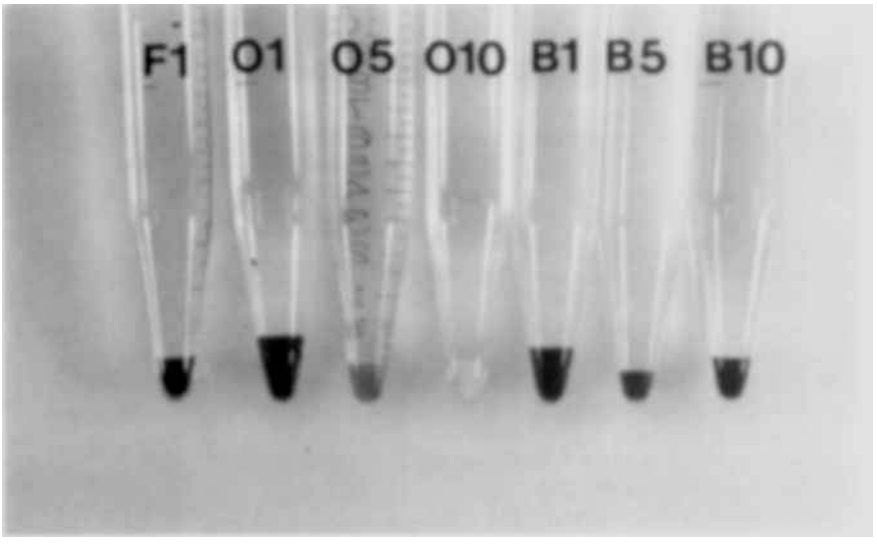

Fig. 9. Relative melanin contents of various B16 melanoma lines shown by pigmentation intensities of cell pellets.

melanoma [30], a UV-induced sarcoma [31], and a vasoformative sarcoma [5, 28] indicate that tumor cell clones differ widely in their metastatic properties, and some rare clones exist that possess highly metastatic phenotypes. Subcloning experiments ruled out the possibility that the in vitro cloning techniques caused the variations in phenotypic properties [30]. The second type of experiment was an attempt to adapt cells to grow at a specific organ location by direct inoculation [16]. This experiment was performed using line B16-F1, which was sequentially adapted to grow in brain for a series of ten cycles of similar durations in vivo and in vitro to the brain-selected line B16-B10N. After ten sequential adaptations for brain survival, adaptation, and growth, the adpated line B16-ICer10 was tested for its ability to metastasize to brain. Line B16-ICer10 was no more effective in colonizing brain after intravenous administration than its original parental line B16-F1, suggesting that adaptation per se is not responsible for generating variants with increased metastatic potential [16]. These data suggest that highly malignant, organ-preferring cells preexist in the unselected parental tumor cell population and that in vivo selection of variant lines yields new populations enriched in cells with highly malignant phenotypes.

ACKNOWLEDGMENTS

We thank A. Brodginski, R. Davis, and D. Welch for assistance. These studies were supported by US Public Health Service National Cancer Institute grant R01-CA-15122 and contract NO1-CB-74153 and a grant (BC-211B) from the American Cancer Society to G.L.N.

REFERENCES

1. Nicolson GL, Poste G: New Engl J Med (Part 1) 295:197, 1976, (Part 2) 295:253, 1976.
2. Nicolson GL: Biochim Biophys Acta 458:1, 1976.

3. Zeidman I: Cancer Res 17:157, 1957.
4. Fidler IJ: In Becker FF (ed): "Cancer: A Comprehensive Treatise," vol 4: "Biology of Tumors: Surfaces, Immunology, and Comparative Pathology." New York: Plenum, 1975, p 101.
5. Nicolson GL: Bio Science 28:441, 1978.
6. Fidler IJ: J Natl Cancer Inst 45:773, 1970.
7. Proctor JW, Auclair BG, Rudenstam CM: Int J Cancer 18:255, 1976.
8. Fidler IJ: In Day SB et al (eds): "Cancer Invasion and Metastasis." New York: Raven, 1977, p 277.
9. Dunn TB: J Natl Cancer Inst 14:1281, 1954.
10. Potter M, Rahey JL, Pilgrim HI: Proc Soc Exp Biol Med 94:327, 1957.
11. Parks RC: J Natl Cancer Inst 52:971, 1974.
12. Fidler IJ, Nicolson GL: J Natl Cancer Inst 57:1199, 1976.
13. Fidler IJ, Nicolson GL: J Natl Cancer Inst 58:1867, 1977.
14. Nicolson GL, Brunson KW: Gann Monogr Cancer Res 20:15, 1977.
15. Brunson KW, Beattie G, Nicolson GL: Nature 272:543, 1978.
16. Brunson KW, Nicolson GL: In Weiss L, Gilbert H, Posner JB (eds): "Brain Metastasis." Boston: GK Hall & Co. (in press).
17. Brunson KW, Nicolson GL: J Natl Cancer Inst 61:1499, 1978.
18. Conley FK: Cancer Res 39:1001, 1979.
19. Zeidman I: Cancer Res 21:38, 1961.
20. Fisher B, Fisher ER: Cancer Res 27:421, 1967.
21. Fidler IJ: Eur J Cancer 9:223, 1973.
22. Liotta LA, Kleinerman J, Saidel GM: Cancer Res 36:889, 1976.
23. Nicolson GL, Winkelhake JL, Nussey AC: In Weiss L (ed): "Fundamental Aspects of Metastasis." Amsterdam: North-Holland, 1976, p 291.
24. Gasic GJ, Gasic TB, Galanti N, Johnson T, Murphy S: Int J Cancer 11:704, 1973.
25. Fidler IJ: Cancer Res 35:218, 1975.
26. Fidler IJ, Bucana C: Cancer Res 37:3945, 1977.
27. Nicolson GL, Winkelhake JL: Nature 255:230, 1975.
28. Nicolson GL, Brunson KW, Fidler IJ: Cancer Res 38:4105, 1978.
29. Fidler IJ: Nature New Biol 242:148, 1973.
30. Fidler IJ, Kripke ML: Science 197:893, 1977.
31. Kripke ML, Gruys E, Fidler IJ: Cancer Res 38:2962, 1978.