The Selection and Characterization of an Invasive Variant of the B16 Melanoma

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Several *in vitro* properties of two variant cell lines of the B16 melanoma (B16-F10 and B16-BL6) with markedly different spontaneous metastatic behavior were examined. The two cell lines were compared with regard to their *in vitro* growth rate, ability to migrate, ability to adhere to a variety of substrata, detachment rates, production of plasminogen activator, and cell surface proteins as determined by lactoperoxidase-catalyzed iodination. Growth rates *in vitro*, attachment rates, and qualitative patterns of cell surface proteins were almost identical. B16-F10 cells (the less spontaneously metastatic line) produced greater amounts of plasminogen activator, were more motile *in vitro*, and detached more readily from plastic than the more invasive B16-BL6 cells. The study of tumor cell variants, selected for different biologic behavior, is a valuable approach to the elucidation of those mechanisms reponsible for their malignant activity. (Am J Pathol 97:587-600, 1979)

THE INFILTRATION AND INVASION of host tissue by malignant cells is a vital step in the pathogenesis of metastasis. Before a primary tumor can disseminate, it must invade either lymphatic or blood vessels in order to release individual cells or cellular aggregates into these channels. Failure to penetrate these vessels will result in the localization of the primary tumor and render it more amenable to such therapeutic interventions as surgery or x-irradiation. Likewise, once tumor cell emboli have lodged in the capillaries of distant organs, the establishment of secondary neoplastic foci is dependent on the penetration of the vessel wall and infiltration into the surrounding parenchyma.

In spite of the fundamental importance of tumor cell invasion, relatively little is known about the mechanisms responsible for this phenomenon. The proposed mechanisms are summarized in Table 1. These mechanisms, for which there is only indirect evidence, need not be mutually exclusive, may vary in relative importance between tumor systems, and may be modified by such factors as anatomic site or host response.

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A possible approach to the identification of those tumor cell properties that allow for successful invasion is to study variants of the same tumor with differing invasive capacities. If tumor cell populations are heterogeneous with regard to their metastatic behavior,[1-4] then it might be expected that subpopulations of highly invasive cells preexist within the parent population of a primary tumor. Application of the correct selection pressure to the starting population of tumor cells would lead to the isolation of the desired variant. Those properties thought to be essential for invasion could then be studied in the variant and the parent and compared to each other. Using this approach with the murine T-241 fibrosarcoma, Liotta et al [5] recovered cells from the tumor venous drainage, presumptively the invasive population, and found they solubilized pulmonary basement membrane from syngeneic mice to a significantly greater extent than cells from the primary tumor mass. In this report, we compare certain cellular properties of two B16 melanoma variants of differing invasive capacity.

Materials and Methods

Animals

Specific-pathogen-free C57BL/6 mice were obtained from the Animal Production Area of the Frederick Cancer Research Center. Within a single experiment the mice were sex- and age-matched.

The parent tumor cell population used in this study was the B16-F10 cell line, selected for its lung colonizing ability following intravenous injection.[1] The selection of the invasive variant derived from this cell line is described elsewhere.[6] Briefly, B16-F10 cells in single cell suspension were injected into the urinary bladder of male C57BL/6 mice via the vas deferens. The bladder was ligated, excised, and maintained *in vitro* on semisolid

Table 1—Proposed Mechanisms of Tumor Cell Invasion

A. Tumor cell properties
   1. Rapid cellular proliferation leading to infiltration via mechanical pressure
   2. Reduced adhesiveness—enhanced detachment from the primary tumor
   3. Cell motility
      a. Translocative—tactic or nontactic response
      b. Stationary—disruptive effect, eg, cytoplasmic projections
   4. Degradative action of lytic enzymes
      a. Tumor-derived—either secreted or cell-surface-associated

B. Host responses
   1. Inhibitory
      a. Immunologic
      b. Mechanical barrier
   2. Enhancing
      a. Immunologic, eg, migrating tumor cells in wake of leukocytes
      b. Vascularization
      c. Degradative enzymes, eg, neutrophils, macrophages

agar and incubated in a humidified atmosphere, 5% CO_2 at 37 C. Tumor cells that migrated through the wall of the bladder were recovered from the agar, cultured, and repassaged. This process was repeated six times, and the resulting variant was designated B16-BL6. B16-F10 was recovered from the agar after 6 days' culture of the organ explant; B16-BL6 was recovered 1 day after the cells from the previous passage had been inoculated into the bladder.

Tumor Cultures

The cultures were maintained on plastic in Eagle's minimal essential medium supplemented with 10% fetal calf serum (FCS), sodium pyruvate, nonessential amino acids, L-glutamine, and two-fold vitamin solution (Flow Laboratories, Rockville, Md). This medium was termed complete minimal essential medium (CMEM). The cell lines were examined for and found free of Mycoplasma and the following murine viruses: reovirus type 3, pneumonia virus of mice, K virus, Theiler's virus, Sendai virus, minute virus of mice, mouse adenovirus, mouse hepatitis virus, lymphocytic choriomeningitis virus, ectromelia virus, and lactate dehydrogenase virus (Microbiological Associates, Walkersville, Md). Cultures were grown at 37 C in a humidified atmosphere containing 5% CO_2.

Tumor Cell Dissemination and Growth Following Subcutaneous or Intramuscular Injection

B16-F10 and B16-BL6 melanoma cells were harvested from nonconfluent monolayers by a 2-minute incubation with 2 mM EDTA solution in $Ca^{++}Mg^{++}$-free phosphate-buffered saline (PBS). The cells were washed once, and the suspension was adjusted to 5.0×10^5 viable cells/ml Hanks' balanced salt solution (HBSS). Viability was assessed by trypan blue exclusion, and only suspensions containing >95% viable single cells were used. C57BL/6 mice were injected subcutaneously in the external ear with 0.1 ml tumor cell suspension or intramuscularly in the footpad of the hind leg with 0.05 ml tumor cell suspension as detailed in the Results section. After a predetermined time, the mice were killed and autopsies were performed. The number of spontaneous metastases found in the lungs were counted under a dissecting microscope.

In Vivo Growth Rate

Mice injected intramuscularly in the footpad or subcutaneously in the ear were examined at weekly intervals for tumor growth. The latent period before tumor growth and the mean diameter of the growing tumor in millimeters were measured.

In Vitro Growth Curves

Cells from both variant lines were harvested as detailed above and plated at a density of 2.5×10^4 cells/60-mm dish in 5 ml CMEM. Cultures were incubated at 37 C in a humidified atmosphere containing 5% CO_2. Triplicate samples were terminated 24, 48, 72, and 96 hours after plating. The cells were harvested using 0.25% trypsin and 0.02% EDTA and counted in a Coulter Counter, Model 2B1 (Coulter Electronics, Inc., Hialeah, Fla). Cultures were refed at 48 and 72 hours.

In Vitro Migratory Activity

The technique of Varani et al [7] was used to measure the *in vitro* migratory activity of the two cell lines. Subconfluent cultures of both cell lines were harvested and adjusted to 1×10^6 viable cells/ml CMEM. Five-milliliter aliquots of the cell suspensions were pipetted into plastic culture tubes and centrifuged at 400g for 10 minutes. The cell pellets were then agitated with 0.2% Noble agar in CMEM (0.3 ml of agar to each 0.1-ml cell pellet). A

1-μl drop of agar/cell mixture was placed in the center of an individual well of a Microtiter II dish (Becton, Dickinson and Co., Oxnard, Calif), and the whole dish was chilled at 4 C for 10 minutes to allow the agar to solidify. Prechilled CMEM (0.1 ml) was added to each well, and the cultures were incubated at 37 C. At 24, 48, 72, and 96 hours, individual wells were examined to determine the cell migration that had occurred during that time. The distance between the leading edge of the migrating cells and the edge of the agarose droplet was measured with an inverted microscope equipped with a linear micrometer (American Optical, Buffalo, NY) in the eyepiece. Four wells for each cell line were monitored at each time point (the same wells for the duration of the experiment), and the measurements were obtained from the four sides of each droplet.

Plasminogen Activator Production

B16-F10 and B16-BL6 cells were harvested as above, and 2.5×10^4 viable cells were plated into 30-mm Petri dishes. Duplicate samples for each cell line were grown to either subconfluency or complete confluency. For the assay of plasminogen activator (PA) production, the incubating medium was replaced with 1 ml serum-free CMEM for 24 hours. After 24 hours, the medium was collected, centrifuged at 400g for 10 minutes, and the cell-free supernate was assayed for secreted PA. The cells were lysed by the addition of 1 ml 0.1% Triton X-100 in phosphate-buffered saline, and then the suspension was homogenized in an ice-cold ground glass homogenizer (30 strokes) before the cell extract was assayed for PA activity. The protein content of cell extracts, medium, and plasminogen solutions was determined by a modified Lowry technique.[8] Duplicate cultures were terminated and cells were counted in a Coulter Counter as described above.

Fibrinolysis Assay

The assay has been described in detail elsewhere.[9] Briefly, Linbro Disposo trays were coated with ^{125}I-labeled fibrinogen and dried for 3 days at 37 C so that final fibrinogen concentration in the wells was 10μg/sq cm and each well contained $2\text{–}3 \times 10^5$ cpm. Fibrinolysis assays contained up to 20 μl of cell extract or serum-free harvest fluid as a source of PA and 5 μg human plasminogen (obtained by lysine–Sepharose 4B affinity chromatography of human serum) (Gibco, Grand Island, NY) in a total volume of 250 μl tris-HCl buffer (0.1 M, pH 8.1). After 3 hours incubation, the supernate was removed and counted to determine the counts per minute (cpm) of released ^{125}I.

Adhesion Rates

Adherence rates of B16-F10 and B16-BL6 cells to monolayers of bovine aorta cells and B16-F10 cells were determined. Aorta cells or B16 melanoma cells were plated into multiwell dishes (Costar, Cambridge, Mass) and allowed to grow to confluency. Culture supernates were removed by aspiration, and 1 ml CMEM was added to each well; the monolayer culture was then incubated for 60 minutes more. Both cell lines were labeled with ^{125}I-iododeoxyuridine (^{125}IUdR) as previously described.[10] The ^{125}IUdR-labeled cell lines were harvested and adjusted to 5×10^5 cells/ml; the adherence assay was performed by the addition of 0.1 ml of tumor cell suspension to each well and incubation of the dishes. Triplicate samples were terminated at 5, 10, 20, 30, 45, and 60 minutes by aspiration of the supernates and nonadherent cells. The cultures were rinsed twice with PBS, and the remaining adherent cells were lysed by the addition of 0.1 ml 1N NaOH. The lysates were collected and monitored for radioactivity, as were three representative input doses from both cell lines. From these values the percentage of adherent cells was calculated for each time point.

Detachment Assay

B16-F10 and B16-BL6 cells in the exponential phase of growth were labeled with ^{125}IUdR. Cells from both lines were harvested as described above, and an aliquot of cell suspension containing 1×10^4 cells was added to 1 ml CMEM in individual wells of cluster dishes. Similar aliquots were taken and monitored for radioactivity to determine input values. The dishes were incubated at 37 C for 24 hours. Following incubation, the adherent cells were washed twice with PBS to remove floating cells, and then 1 ml of 0.25% trypsin and 0.02% EDTA solution was added to each well. The whole dish was placed on an orbital shaker platform (Bellco Glass, Vineland, NJ) at room temperature (23 C). At time points of 1, 5, 10, 20, 30, and 45 minutes, the trypsin solution plus detached cells was aspirated from triplicate wells and monitored for radioactivity. From these values the number of detached cells was calculated for each time point as a percentage of the total added.

Lactoperoxidase Radioiodination of Tumor Cell Membrane Proteins

The technique of Marchalonis et al [11] as modified by Haustein [12] was used to radioiodinate tumor cell surface membrane proteins. Both B16-F10 and B16-BL6 tumor cell lines, maintained on plastic as described above, had the incubating medium replaced with serum-free CMEM and were incubated for 24 hours more. Both cell lines were then harvested as described above, washed three times with cold PBS, and adjusted to 4×10^6 cells in a test tube. The cells were pelleted by centrifugation, and to each pellet was added 30 μl PBS, 10 μl lactoperoxidase (0.25 mg/ml PBS), 5 μl ^{125}I as iodide (100 mCi/ml) (Amersham Searle, Des Moines, Ill), and 10 μl hydrogen peroxide (0.03%). The cells were held at 30 C. At 3-minute intervals the additions of lactoperoxidase and of hydrogen peroxide were repeated three times, and hydrogen peroxide alone was added for the final 4-minute incubation. The cells were then washed once with 10 ml cold PBS.

Extraction of Labeled Cell Surface Proteins

Iodinated tumor cells were pelleted and then lysed by the addition of 1 ml 0.1% Triton X-100 for 2 hours at 23 C. The solutions were recentrifuged at $400g \times 10$ minutes to remove nuclei and debris, and the supernates were dialyzed against tris-buffered saline (TBS, pH 8.0 : 0.05 M tris (hydroxymethyl)aminomethane, 0.15 M NaCl) overnight at 4 C. Labeled proteins were analyzed by sodium dodecyl sulfate polyacrylamide gel electrophoresis (SDS–PAGE) as previously described.[12] Gels were fixed, frozen on dry ice, and sliced. The slices were monitored for radioactivity with the use of a Searle automatic well-type gamma scintillation spectrometer.

Results

Spontaneous Metastatic Behavior of B16 Variants B16-F10 and B16-BL6

Data from two representative experiments are presented in Table 2. The line B16-BL6, selected for increased invasive capacity, consistently produced greater numbers of pulmonary metastases in syngeneic mice than the parent line when injected intramuscularly or subcutaneously. In the present experiments 87% of mice given injections of B16-BL6 cells developed spontaneous pulmonary metastases, whereas 33% of the mice

Table 2—Spontaneous Metastatic Behavior of B16 Variant Lines Injected into C57BL/6 Mice

	Tumor line	Number of mice bearing pulmonary tumors	Number of pulmonary tumors
Experiment 1—intramuscular site*	F10	3/9	2,7,11
	BL6	8/9	23,59,81,86,150 180, >500, >500
Experiment 2—subcutaneous site†	F10	3/9	2,2,29
	BL6	6/7	1,1,19,26,34,72

* The mice were given injections in the footpad of 25,000 cells in 0.05 ml HBSS. The leg was amputated 4 weeks later. The mice were allowed to survive a further 4 weeks; then autopsies were performed.

† The mice were given injections in the external ear of 50,000 cells in 0.1 ml HBSS and left for 4 weeks; then autopsies were performed.

given injections of equal numbers of B16-F10 cells in similar sites developed spontaneous metastases to the lungs.

In Vivo and *In Vitro* Growth Rates

The *in vitro* growth curve of the two variant lines is given in Text-figure 1. Both B16-BL6 and B16-F10 cells had doubling times of 12–13 hours.

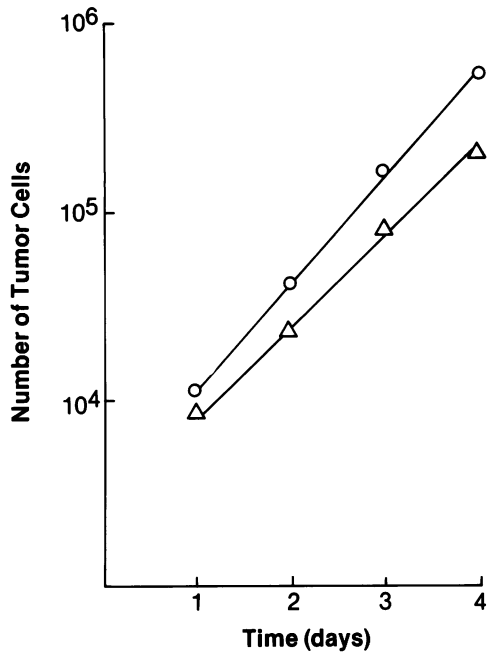
Patterns of subcutaneous or intramuscular growth failed to show any differences between the two cell lines (Text-figure 2); the length of latent period and the final size of the tumor at the time of autopsy were the same for both variants.

In Vitro Migratory Activity

The *in vitro* migratory rates of B16-F10 and B16-BL6 cells are presented in Text-figure 3. In 7 of 7 experiments B16-F10 cells consistently showed greater *in vitro* motility than B16-BL6 cells, although the locomotory rates were not always significantly different. In the experiment shown, the distance migrated by the two cell lines was significantly different (Student t test, $P \leq 0.001$) on Days 2, 3, and 4.

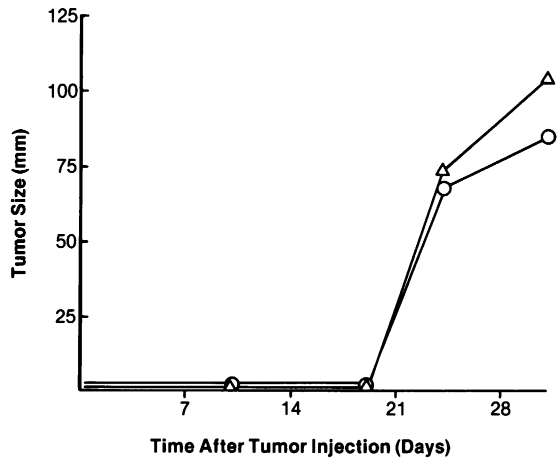
Plasminogen Activator Production

The plasminogen-dependent release of ^{125}I counts per minute from the ^{125}I-fibrin-containing well as a function of cell extract (expressed as numbers of cells added) obtained from confluent cultures is shown in Text-figure 4. Similar patterns were obtained when the source of PA was conditioned medium harvested from either confluent or subconfluent cultures or cell extracts from subconfluent cultures. Equally, expression of enzyme

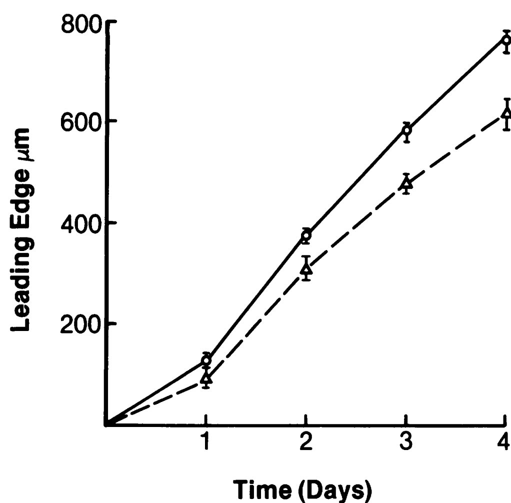


TEXT-FIGURE 1—*In vitro* growth curves of B16 variant cell lines. Cells were plated at a density of 2.5×10^4 cells/60-mm dish. ○——○, B16-F10; △——△, B16-BL6.

activity as a function of milligrams protein added gave curves identical to those of expression of enzyme activity as a function of cell numbers added. In all assays performed, B16-F10 cells produced and secreted higher levels of PA than B16-BL6 cells.



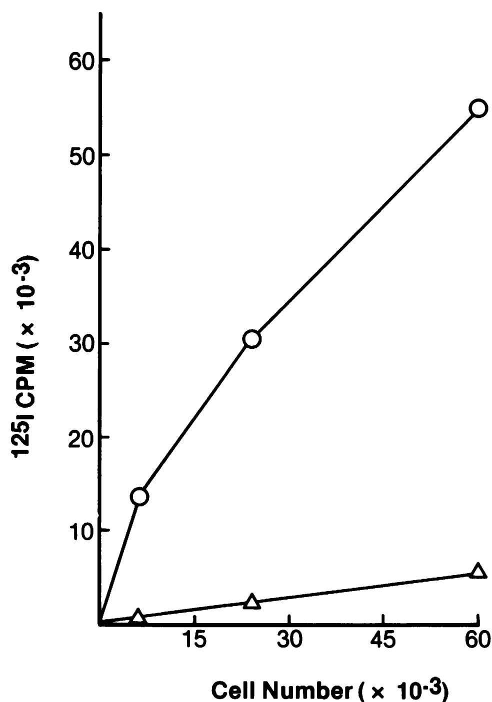
TEXT-FIGURE 2 — *In vivo* growth curves of B16 variant cell lines. C57BL/6 mice (9 per group) were given intramuscular injections in the footpad of 25,000 cells of either B16-F10 (○——○) or B16-BL6 (△——△).



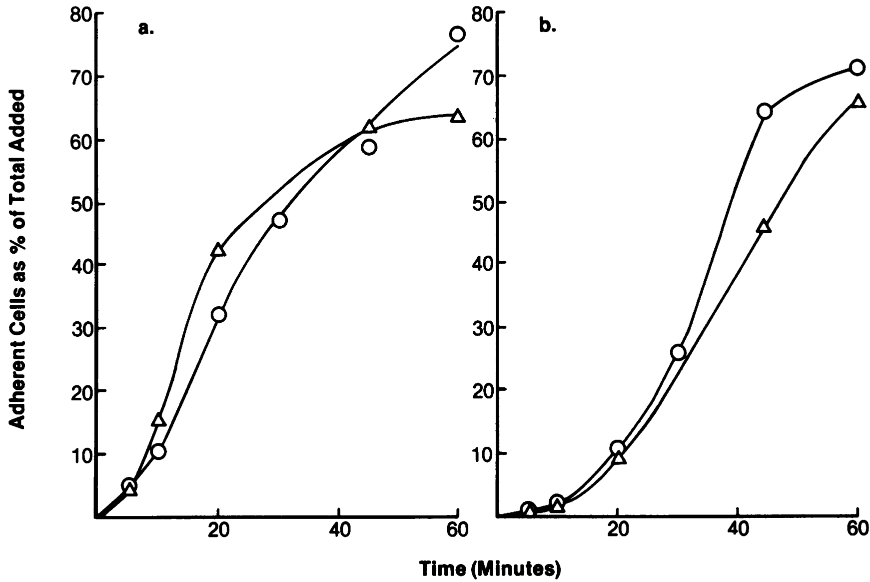
TEXT-FIGURE 3—Migration of B16 cells beyond the edge of agarose drop explants. O——O, B16-F10 cells; Δ——Δ, B16-BL6 cells. Each point represents mean ± SD of 16 estimations (4 drops, 4 readings/drop).

Adhesion Rates

The adhesion rates of B16-F10 and B16-BL6 cell lines to a variety of substrata (serum-coated plastic, 3T3 fibroblasts, bovine aorta cells, TSII epidermal cells, and B16 melanoma cells) are very similar, although



TEXT-FIGURE 4—Cell-associated levels of plasminogen activator of B16 variant cell lines. Activity expressed as release of ^{125}I counts per minute per number of cells in extract harvested from confluent cultures. O——O, B16-F10; Δ——Δ, B16-BL6.



TEXT-FIGURE 5—Adhesion rates of B16 variants to bovine aorta cells (*a*) and B16 melanoma cells (*b*) in monolayer culture. O——O, B16-F10; Δ——Δ, B16-BL6. Each point represents the mean of triplicate samples.

only the kinetics of adhesion to bovine aorta cells and B16 monolayers are presented here. Results from a typical experiment are presented in Text-figure 5.

Detachment Rates

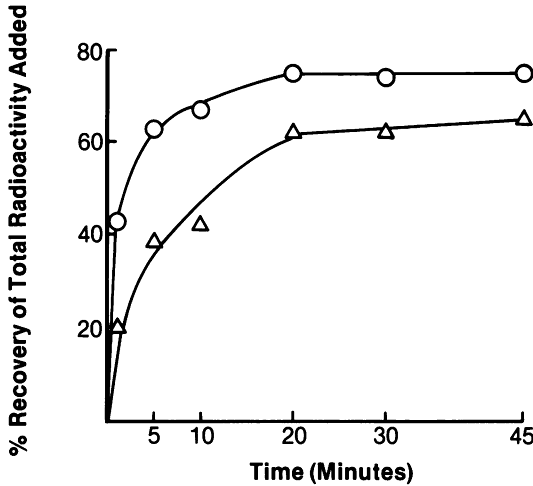
In three experiments, B16-F10 cells detached from serum-coated plastic under the influence of trypsin more rapidly than BL6 cells (Text-figure 6).

Labeling of Surface Proteins

Separation of ^{125}I-labeled cell surface proteins by SDS–PAGE failed to reveal any qualitative differences between the two cell lines (Text-figure 7), although there may be minor quantitative differences.

Discussion

The ability of malignant cells to invade surrounding host tissue and disseminate to distant sites has been attributed to many factors (Table 1). Cellular properties such as rapid growth rate, reduced adhesiveness, active cell motility, and the production of degradative enzymes may play an important role in determining the outcome of metastasis. By comparing

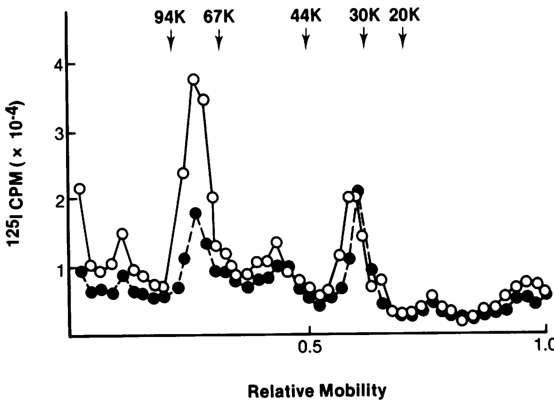


TEXT-FIGURE 6—Detachment rates of B16 variants. Detachment from serum-coated plastic induced by 0.15% trypsin-0.02% EDTA solution. ○——○, B16-F10; △——△, B16-BL6. Each point represents the mean of three individual experiments.

variant cell lines of different invasive capacity with regard to those characteristics, we have attempted to determine the factors that serve as invasive mechanisms.

Rapidly dividing, anaplastic tumors are frequently more invasive than better differentiated tumors,[13] and it may be that the pressure generated by such cellular proliferation forces finger-like processes of cell cords along lines of least resistance.[14] However, many studies suggest that rapid cell growth is not a vital prerequisite of invasive growth,[15,16] and indeed this would appear to be so for the B16 melanoma. The similarity of *in vitro* and *in vivo* growth patterns of the two variant cell lines makes it unlikely that the enhanced spontaneous metastatic behavior of BL6 is due to increased cellular proliferation.

A possible explanation for tumor cell invasion is that cells with greater



TEXT-FIGURE 7—Radioiodination of cell surface proteins of B16 variants. Labeled proteins were analyzed by SDS-PAGE under reducing conditions (5% 2-mercaptoethanol in the sample buffer) on gels of 10% acrylamide, 0.15% bisacrylamide. Arrows (↓) indicate the mobility of external standards.

malignant potential have an increased translocative motility that facilitates their spread through host tissues. There have been few studies in which the translocative movement of cell lines is compared with their differing malignant behavior, but Gershman and his colleagues [17] have correlated an increased tumorigenic potential with increased cell mobility in hamster NIL-B cells transformed with SV40 virus. The *in vitro* motility of the B16-F10 and B16-BL6 lines was assayed by the technique of Varani et al.[7] Instead of finding that the more spontaneously metastatic cell line (BL6) was the more motile, we found that B16-F10 cells consistently migrated at a faster rate than B16-BL6 cells. It has previously been reported that normal mouse embryo fibroblasts are more motile than mouse fibrosarcoma cells when assayed within this system,[7] whereas variant cell lines established from the same tumor do not show a correlation between increased motility and more malignant behavior.[18]

A variety of tumorigenic and transformed cells have been shown to produce high levels of the serine protease PA.[19] Since increased fibrinolysis may facilitate tumor penetration,[20] the association between tumor cell invasion and PA production warrants investigation. Using the two variant cell lines, we were unable to correlate increased invasion with increased PA activity. Cells from the B16-BL6 line secreted PA or had cell-associated PA at considerably lower levels than cells from the B16-F10 line. Many nontumorigenic cells produce high levels of PA activity *in vitro*,[19] and if the formation of fibrin around a tumor embolus acts as a protective mechanism,[21] it may even be that fibrinolysis does not enhance tumor dissemination. Nicolson and colleagues [22] found no differences between two other variant lines (B16-F10 and B16-F1) with regard to PA production, and our findings support their contention that malignancy of B16 melanoma variants is independent of PA production.

The concept that malignancy is associated with reduced cell adhesiveness has been assimilated into the clinical literature.[23] However, in our studies there were no differences in the rates of adhesion to a variety of substrata between the cell lines, whereas B16-F10 cells were detached more readily from a serum-coated plastic substratum in the presence of trypsin than B16-BL6 cells.

Because many interactions between malignant and host cells are probably modified by the nature of the tumor cell surface, we thought it necessary to compare the cell surface composition of the variant lines. In this investigation we used the technique of lactoperoxidase-catalyzed iodination to examine the exposed proteins of the cell surface of the B16-BL6 and the B16-F10 cells. No qualitative differences were found between the

two variant lines, but it did appear that there may be possible quantitative differences. A correlation between increased selection and metastatic potential and modification of cell surface proteins has been reported for the B16 melanoma,[24] but the quantitative nature of this protein-labeling technique has been questioned.[25]

In order to determine those cellular properties that are important for expression of the malignant phenotype, we compared cell lines of different biologic behavior isolated from the same tumor. The need to refer to normal cells, often of doubtful validity, is thus obviated, while a basis for comparison is still maintained. To date, many differences between so-called transformed and non-transformed cells have been reported,[26] but the relevance of these findings to the metastatic process is questionable. Three methods of isolation of tumor cell variants are available. Tumor variants may be selected *in vitro* for specific properties, such as motility or detachment from a monolayer,[27] and their metastatic potential *in vivo* may then be assessed. Alternatively, variant lines of different metastatic potential may be selected and then compared with regard to their *in vitro* properties, as we have done in this study. Finally, a large number of clones may be established *in vitro* [3,4] and then examined for varying *in vitro* and *in vivo* behavior. The studies reported here have examined *in vitro* characteristics of two cell lines with markedly different *in vivo* properties. In the system chosen we have been able to show many differences between the two variant lines but have been unable to correlate or determine the relevance of those findings to the *in vivo* behavior of the two cell lines. *In vitro* assays are, of necessity, oversimplified models of likely *in vivo* conditions. Attachment to or motility over artificial substrata may not be at all comparable to reactions within the body; the finding of peaks or bands on gel electrophoresis is of little relevance until we can determine the nature and role of such components in the metastatic sequence; kinetics of attachment and detachment do not measure the actual forces between tumor cells or between tumor cells and other tissues. Nonetheless, the utilization of variant lines allows us to compare biologically relevant cells; and although the work may be tedious, the likelihood of finding properties of significance in malignant behavior is increased. We are aware that the amplification of various characteristics by selection, as described here and elsewhere,[1,28,29] still may not be sufficient to allow for identification of the underlying mechanisms, but we feel that such an approach is the most promising way to attempt to unravel those tumor properties necessary for malignant behavior.

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