In Vitro Selection of Murine B16 Melanoma Variants with Enhanced Tissue-invasive Properties

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ABSTRACT

New assay methods have been devised to quantitate tumor cell invasion of tissues of differing histological complexity maintained as organ cultures in vitro (chorioallantoic membrane of chicken, mouse urinary bladder, and canine blood vessel). In addition to quantitating tumor cell invasion, these methods also allow recovery of invasive cells for comparison with noninvasive cells. These methods have been used to select variant sublines from murine B16-F1 and B16-F10 melanoma lines that display significantly greater tissue-invasive abilities than the parent lines. B16 variant sublines selected in vitro for increased invasiveness through the bladder wall or vein also show a significant increase in their ability to form spontaneous and experimental metastases in vivo. In contrast, cells from the same parent cell line selected for increased invasiveness through the chorioallantoic membrane do not show significant alterations in metastatic behavior. We conclude that invasive variants can be isolated from the parent B16 tumor by several in vitro methods and that the level of expression of the invasive phenotype in vivo may be determined by the severity of the selection procedure in vitro.

INTRODUCTION

Invasion of lymphatics and blood vessels by malignant cells provides a major pathway for the dissemination of neoplastic cells within the body (for reviews, see Refs. 7, 8, and 27). In addition, following their arrest in the capillary beds of different organs, circulating tumor cells invade the wall of these vessels and escape into the extravascular tissue(s) where they establish metastases. Despite its obvious importance in the metastatic process, relatively little is known about the mechanism(s) of tumor invasion. This deficiency reflects the formidable technical problems encountered in studying invasion in vivo and the lack of quantitative methods for studying invasion in vitro and for recovering invasive cells once invasion has taken place.

A substantial body of evidence has been assembled in the last few years which indicates that primary malignant neoplasms are not homogeneous entities of cells with uniform properties but instead contain subpopulations of tumor cells with widely differing metastatic abilities (for reviews, see Refs. 7, 8, and 27). This phenotypic heterogeneity dictates that examination of heterogeneous unselected tumor cell populations may offer little insight into the cellular properties responsible for invasion and/or metastasis if only very few cells within the population express these behavioral traits. A more productive approach to the experimental analysis of the cellular properties needed for successful invasion is to isolate invasive tumor cell subpopulations and compare them with poorly invasive or noninvasive tumor cells derived from the same parent cell population. In this report, we describe methods for the in vitro isolation of a series of murine B16 melanoma cell variants with enhanced invasive properties and an initial characterization of their behavior in vivo.

MATERIALS AND METHODS

Cells. The origin and properties of the B16-F1 (low potential for lung colonization) and B16-F10 (high potential for lung colonization) sublines of the B16 melanoma have been described in detail previously (5, 12). Cultures of mouse embryo fibroblasts were prepared by trypsinization of 14- to 16-day-old C57BL/6 mouse fetuses as described elsewhere (29). Homogeneous peritoneal macrophage cultures were obtained from C57BL/6 mice inoculated i.p. with sodium thioglycollate (Baltimore Biological Laboratories, Cockeysville, Md.) as described previously (29). All cells were incubated in plastic flasks or Petri dishes in MEM* (12, 29). The components of CMEM were obtained from Flow Laboratories, Inc., Rockville, Md., and the Grand Island Biological Co., Grand Island, N. Y. Cell cultures were incubated at 37°C in a humidified atmosphere containing 5% CO2 in air. All cultures were free of Mycoplasma species and pathogenic murine viruses (12), and they remained so throughout the experiments. In certain experiments, cell cultures (except macrophages) were labeled with [125I]I]UdR as detailed in Ref. 5. Briefly, actively growing nonconfluent monolayer cell cultures were incubated for 24 hr in CMEM containing [125I]I]UdR (0.2 μCi/ml; specific activity, 200 μCi/mmol; New England Nuclear, Boston, Mass.). This method labels more than 95% of the cells and does not alter their metastatic behavior in vivo (5).

Assay of Tumor Cell Invasion in CAM and Selection of Invasive Cell Variants. Newly fertilized eggs were incubated at 38°C in a humidified atmosphere (relative humidity, approximately 75%) in an egg incubator (Favorite Incubator; Leaky Mfg. Co., Higginsville, Mo.) until Day 8 after fertilization, when the CAM was dropped using the false air sac method as described elsewhere (28). Two days later, the CAM was harvested and dissected by a sterile technique into pieces approximately 0.625 inch square for eventual transfer to the...
Selection of Invasive Variants

invasion chamber. The latter, shown in cross-section in Chart 1, comprises an upper and lower chamber constructed from 2 sterile Teflon rings. The edges of these rings were rendered smooth by heating before use. To prepare the chamber, we placed the lower ring (o.d., 0.375 inch; i.d., 0.25 inch; height, 0.375 inch) in a sterile plastic Petri dish (100-mm diameter), and a 0.25-inch-thick layer of sterile nutrient agar was poured around the ring for support. Nutrient agar was prepared as described previously (28). The inside of the lower ring was then filled with nutrient agar, and a dissected piece of CAM was placed over the lower ring (ectodermal surface facing up) after which the larger upper ring (i.d., 0.375 inch; height, 0.5 inch) was placed in position to trap the CAM. A second layer of semisolid support agar was then poured into the dish around the upper ring so that the 2 rings, plus the free edges of the CAM, were held in a firm agar support (Chart 1). B16 melanoma cells (1 x 10⁶) labeled with I²⁵I-labeled were then added to the upper chamber as a single-cell suspension in CMEM, and the chamber was incubated at 37° for up to 7 days. The structural integrity of the CAM was measured at the end of each assay by aspirating the culture medium from the upper chamber (this is not discarded; see below) and replacing it by 0.4 ml India ink (Pelikan, Hannover, W. Germany). Only the chambers retaining the ink in the upper compartment for at least 30 min were considered acceptable. Preparations showing leakage of ink to the bottom compartment were rejected. With completion of this test, the upper ring and the CAM were removed from the chamber. The I²⁵I radioactivity in the CAM and in the culture medium aspirated from the upper compartment (see above) was measured with a Packard gamma counter. The I²⁵I radioactivity of the contents of the lower compartment was then measured and expressed as a percentage of the total radioactivity in the upper and lower compartments and the CAM. This figure represents the percentage of cells in the original cell inoculum which had successfully penetrated the full thickness of the CAM. Several chambers can be prepared in the same dish (Fig. 1) permitting replicate cell populations to be tested in parallel.

For selection of tumor cell variants with increased invasive properties, cells added to the upper compartment are not radiolabeled. After cells were incubated in the chamber for 7 days, the integrity of the CAM is tested using India ink as described above, after which the upper ring and the CAM are removed and discarded. Cells that had penetrated the CAM and grew as colonies in the agar in the bottom compartment (Fig. 2) were then harvested and cultured further in plastic flasks to obtain a sufficient number of cells, and 1 x 10⁶ viable cells were inoculated onto fresh CAM preparations. The cells

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Fig. 1. Petri dish containing 4 CAM invasion chambers (described in Chart 1) embedded in agar. Pigmented B16 melanoma cells can be seen growing in the individual chambers.

Fig. 2. Individual CAM invasion chamber embedded in agar in which pigmented colonies of B16 melanoma cells on the CAM can be seen. By focusing at different levels, colonies could also be identified below the CAM in the agar in the bottom chamber.
penetrating the new CAM's were, in turn, harvested and passed again through fresh CAM's up to 14 times. These cells will be described by the prefix CP (CAM-passage) followed by the number of times the cells had been passed through CAM (e.g., B16-F1-CP10 = B16-F1 cells passaged 10 times through the CAM).

In most selection experiments, nutrient agar was used in the lower chamber. Normal CAM cells were unable to grow in agar, but B16 tumor cells could form colonies in agar because of their loss of anchorage dependence. In certain experiments, fluid culture medium (CMEM) or factors which are chemotactic for macrophages or leukocytes and/or certain tumor cells were placed in the bottom compartment to determine if penetration of radiolabeled tumor cells was affected by such chemotactic stimuli. Bacterial culture filtrates containing leukotactic factors were produced from cultures of Escherichia coli (WHO reference strain 16) as described by Ward et al. (41). Serum fractions containing leukotactic factors were produced by zymosan activation of human serum in the presence of 1 m ε-aminocaproic acid (40). Zymosan-activated sera were incubated with trypsin using the method described by Orr et al. (25) to generate material containing factors from the third and fifth components of complement which are chemotactic for some tumor cells (25). All of the above materials were added to MEM at a final concentration of 75 μl/ml. In other experiments, the bottom chamber was filled with cell-free culture supernatant fluid harvested from the same tumor cell population added to the upper chamber.

Assay of Tumor Cell Invasion in Mouse Urinary Bladder and Selection of Invasive Cell Variants. Adult male C57BL/6 mice were killed by ether inhalation. The mice were submerged in a 5% Wescodyne solution (West Chemical Products, New York, N. Y.), immersed in 70% ethanol for 2 min, and placed into a laminar flow hood. The abdominal cavity was opened in a sterile fashion, and the urogenital system was fully exposed (Fig. 3). Urine within the urinary bladder was expressed by gentle finger pressure (sterile surgical gloves were worn during these procedures), after which the urethra was occluded by application of a hemostat. One testis was displaced from the scrotal sac, and the ductus deferens was identified and spread across an open pair of forceps, with care being taken not to rupture the duct. The ductus deferens was penetrated (Fig. 3) using a 27-gauge needle (Becton, Dickinson, and Company, Rutherford, N. J.) attached to a tuberculin syringe, and 0.3 to 0.4 ml of tumor cell suspension (0.9 to 1.2 × 10⁶ cells) was injected slowly. The injected cells reached the urethra via the emergence of the ductus deferens in the prostate, and retrograde urethral flow carried suspended tumor cells to the bladder which gradually became filled with cells (Fig. 4). A ligature of 2-0 chromic catgut (Detnatel, Queens Village, New York, N. Y.) was placed around the neck of the bladder distal to the site where the base of the bladder had rested was then pipetted up and added to 1 ml CMEM in a single well of a Costar 16-mm multiwell dish (Costar, Cambridge, Mass.). These dishes were incubated under the same conditions as described above, and the wells were examined daily for tumor colonies. When tumor
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Assay of Tumor Cell Invasion in Veins and Selection of Invasive Cell Variants. The principle of the culture system used to isolate tumor cell variants capable of invading the wall of veins is shown in Chart 2. Portions of dog femoral vein were fitted onto a central tube of porous ultrahigh-molecular-weight polyethylene with a pore size of 20 μm (Glassrock Products, Fairburn, Ga.). Culture medium was perfused through the center of this tube from a reservoir by a variable-speed peristaltic pump with flow rates at 2 to 4 ml/min. Tumor cells were injected into the outer chamber surrounding the blood vessel via injection ports fitted with self-sealing rubber caps. The injected cells then attached to the surface of the vein. Segments of vein can be placed around the central tube with either the endothelium facing inwards or as everted segments in which the endothelium is facing the injection ports. Cells that are able to invade the wall of the vein will then penetrate the pores of the central tube and be recovered in the internal perfusion circuit.

The actual equipment is shown in Fig. 5. The main chamber is a modified Amicon Vitaliber Capillary Perfusion System (Amicon, Lexington, Mass.) in which the network of anisotropic...
hollow fiber capillary bundles have been replaced by a 15-cm length of ultrahigh-molecular-weight polyethylene tube. The vein is fitted onto this tube before being placed into the chamber. Segments of dog femoral vein were perfused in situ with ice-cold 0.9% NaCl solution before removal from the animal. Collateral vessels were tied off or occluded by a diathermy ber. Segments of dog femoral vein were perfused in situ with

Collateral vessels were tied off or occluded by a diathermy needle before removing the main portion of vein. Veins were fitted onto the tube by inserting the tube into the lumen of the vessel for a sufficient distance to completely cover the tube. Two encircling catgut ties were then made at each end of the tube, and the excess vein was trimmed with a scalpel. The tube and vessel were then placed into the polycarbonate outer chamber and sealed with screw-fit end pieces and silicon rubber sealing O-rings. Both ends of the tube were then connected to 0.132 inch i.d. x 0.183 inch o.d. gas-permeable medical grade silicone tubing (Dow Corning Corp., Midland, Mich.) to create a closed perfusion circuit with an intervening reservoir of culture medium. The tubing between the reservoir and the input side of the chamber was passed through a 500-ml conical flask containing a mixture of 5% CO2 in air to ensure correct gaseous exchange. Tumor cells (1 x 10⁶) in CMEM (1.5 ml) were then injected into the outer chamber, and the entire apparatus was incubated at 37° in a walk-in hot room. For invasion assays, 125I-IdUrd-labeled cells were injected into the outer chamber, and the number of cells penetrating the wall of the vessel was determined by measuring the radioactivity recovered in the inner perfusion circuit. For selection experiments, nonradiolabeled cells were introduced, and invasive cells harvested from the internal circuit were reinjected into chambers containing fresh vessels. The invasive cell populations recovered by serial passage through veins will be referred to by the prefix BV (blood vessel) followed by the number of times the cells have been passaged through veins.

Animals. Specific-pathogen-free C57BL/6 mice were obtained from the Animal Production Area, Frederick Cancer Research Center, and the West Seneca Laboratories, Roswell Park Memorial Institute.

Metastasis Formation. The ability of cells to form experimental metastases after i.v. injection was measured by injecting 5 x 10⁴ to 5 x 10⁵ viable cells as a single-cell suspension in 0.2 ml HBSS into the tail vein of unanesthetized adult C57BL/6 mice matched for age, weight, and sex. Mice were killed 18 or 21 days later, and the number of lung metastases was determined with a dissecting microscope as described previously (6, 9).

The following methods were used to assay the ability of cells to form spontaneous metastases: (a) C57BL/6 mice were given s.c. injections in the external ear of 5 x 10⁴ cells in 0.1 ml HBSS, and the number of lung metastases was determined 4 weeks later; (b) C57BL/6 mice were given s.c. injections in the ear as above. The ear plus the growing tumor were then amputated 10 days later (7), and formation of regional lymph nodes and lung metastases was determined 4 to 6 weeks later; or (c) C57BL/6 mice were given i.m. injections of 2.5 x 10⁴ viable cells in 0.5 ml HBSS in the footpad of the hind leg. In some animals, the "primary" tumor was amputated after 4 weeks, and metastasis formation was determined 4 weeks thereafter. In other animals, the "primary" tumor was not amputated, and metastasis formation was measured 6 weeks after initial tumor cell injection. In all of the protocols, mice were matched for age, weight, and sex.

RESULTS

In Vitro Selection of B16 Melanoma Cell Variants with Increased Ability to Invade CAM. The ability of B16-F1 melanoma cells to invade and penetrate CAM maintained in vitro and the invasive behavior of variants selected from B16-F1 cells by serial passage of cells that successfully penetrate the CAM are shown in Table 1. The results indicate that the proportion of cells in the parent cell population that penetrate through the CAM to reach the lower chamber (Fig. 2) is very low. Serial passage of the invasive cell fractions recovered from the lower chamber produced a significant increase in the proportion of cells penetrating the CAM. Maximum efficiency of invasion was achieved after 10 passages through the CAM (Table 1).

Confirmation that invasion in this system involves selection (enrichment) of subpopulations with increasing invasive properties was provided by the finding that the "nonpenetrating" cell fractions recovered from CAM's inoculated with early-passage populations contained significantly fewer invasive cells than did the starting inoculum (Table 1). With increasing CAM passage, however, the proportion of invasive cells recovered in the nonpenetrating fraction increased. After 10 passages, the efficiency of invasion by the reinoculated nonpenetrating fraction was similar to that of the original inoculum, suggesting that the cell population was now composed almost exclusively of invasive cells. Thus, in early passages, invasion was a nonrandom process which selects for invasive cell subpopulations. With subsequent passaging, the proportion of invasive cells in the population was enriched; by 10 passages, invasion had become a random process and the population consisted of cells with invasive properties.

The invasive behavior of variants at any given passage level is highly reproducible, and significant variation has not been detected using different CAM preparations. In addition, assays

### Table 1

Invasiveness of B16-F1 mouse melanoma cells during serial passage in chick CAM in vitro

<table>
<thead>
<tr>
<th>Passage no.</th>
<th>% of inoculated cells penetrating the CAM</th>
<th>% of &quot;nonpenetrating&quot; cell fraction penetrating new CAM</th>
</tr>
</thead>
<tbody>
<tr>
<td>1</td>
<td>2.4 ± 0.6</td>
<td>Not detectable</td>
</tr>
<tr>
<td>2</td>
<td>4.1 ± 1.1</td>
<td>1.3 ± 0.4</td>
</tr>
<tr>
<td>4</td>
<td>11.6 ± 2.3</td>
<td>3.9 ± 0.8</td>
</tr>
<tr>
<td>6</td>
<td>17.4 ± 3.7</td>
<td>13.7 ± 2.1</td>
</tr>
<tr>
<td>8</td>
<td>23.7 ± 4.4</td>
<td>18.4 ± 2.7</td>
</tr>
<tr>
<td>10</td>
<td>31.6 ± 4.9</td>
<td>27.6 ± 4.3</td>
</tr>
<tr>
<td>14</td>
<td>30.6 ± 4.2</td>
<td>33.4 ± 4.6</td>
</tr>
</tbody>
</table>

*a* Cell-associated [125I]IdUrd radioactivity recovered in the bottom chamber as a percentage of total radioactivity in the original cell inoculum. Inocula contained between 3.5 and 6 x 10⁶ cpm/10⁶ cells.

*b* Nonradiolabeled cells (1 x 10⁶) were added to CAM invasion chambers and incubated for 7 days at 37° after which "nonpenetrating" cells which failed to reach the bottom chamber were recovered from the culture medium in the upper chamber and from the CAM (see "Materials and Methods"). These were cultured in vitro to generate sufficient numbers of cells, labeled with [125I]IdUrd, and reinoculated onto fresh CAM to assay cell penetration as described in Footnote a.

#c* Mean ± S.E. derived from measurements on 8 replicate chambers at each passage.
of the invasiveness of variants cultivated in vitro for up to 4 months after initial isolation or stored in liquid nitrogen for up to 6 months after initial isolation revealed no significant changes in behavior from that shown in Table 1.

The percentage of cells penetrating the CAM at any given passage level could not be altered significantly by substituting the semisolid agar in the bottom chamber by: (a) fluid culture medium (CMEM) containing different concentrations of fetal calf serum (2, 5, 10, or 20%); (b) cell-free culture supernatant fluids from homologous B16 cell populations; (c) homogenized extract of B16 tumor; or (d) MEM containing components which are chemotactic for leukocytes and macrophages (zymosan-activated serum; E. coli culture fluids) and certain types of tumor cells (trypsin-digested zymosan-activated serum) (results not shown).

Penetration of the CAM requires cells to be viable. $^{125}$I-IdUrd-labeled B16 cells killed by heating at 80° for 15 min before seeding onto the CAM were unable to penetrate. Histological and radioautographic studies revealed that these cells were not able to infiltrate the CAM although they adhered to the surface of ectodermal epithelium. Successful penetration of the CAM also requires more than active cell motility. Studies using macrophages, which are known to infiltrate a wide range of normal tissues, revealed that these cells readily infiltrated the mesoderm of the CAM but did not penetrate the full thickness of the CAM to reach the lower chamber (results not shown).

**In Vitro Selection of B16 Melanoma Cell Variants with Increased Ability to Invade Urinary Bladder Tissue and Veins.** Structurally, the CAM is a relatively simple tissue, and successful invasion does not require tumor cells to breach substantial mechanical barriers such as thick basement membranes, compact fibrous stroma, or muscle layers. Formidable barriers of this kind are routinely encountered by invading tumor cells in vivo (17-22). It was therefore considered worthwhile to see if tumor cell variants could be selected for invasion in tissues containing mechanical barriers of this kind. Urinary bladder and vein were selected since their walls contain all of the barriers listed above. Invasion of blood vessels is also relevant to the pathogenesis of hematogenous metastases in which malignant cells must first penetrate into blood vessels and, after dissemination and arrest in the circulation, cross the wall of blood vessels to reach the extravascular tissues and form metastases (17, 18).

Invasive cell variants have been selected from the B16-F1 melanoma line by selection and passaging of cells which successfully penetrate organ cultures of full-thickness murine urinary bladder wall (Table 2). In contrast to the emergence of invasive variants from B16-F1 cells during passaging with CAM in which the proportion of invasive cells increased relatively slowly with successive passages, the invasive variants selected in bladder emerged much more rapidly. After only 4 passages, the variant population was composed almost exclusively of cells with invasive potential. Although only approximately 25 to 35% of the cells passed 4 or more times penetrated the bladder wall in any given assay (Table 2), the noninvasive fraction in these populations contained large numbers of cells with invasive properties. This was demonstrated by assaying the invasive potential of tumor cells recovered from the lumen of injected bladders. These cells have failed to penetrate the transitional epithelium during initial incubation of cells within the closed bladder. However, when reinoculated into bladders, large numbers of invasive cells could still be detected in this population. As shown in Table 2, after cells have been passaged 4 or more times through the bladder, the invasiveness of "nonpenetrating" cells recovered from the lumen is equivalent to that of the "penetrating" cell fraction (Table 2). This indicates that the population has been enriched for invasive cells which now constitute the majority of cells.

Variant B16 melanoma sublines with increased ability to invade mouse bladder have also been isolated from the B16-F10 line by selecting for cells which are able to penetrate the wall of intact bladders explanted into agar in vitro. The properties of one variant, designated BL6, selected by 6 serial passages through intact bladder will be discussed in more detail later in this paper.

The same strategy used to select B16 melanoma cell variants for increased invasiveness in CAM and bladder has also been successful in selecting variants from the B16-F1 line that display increased abilities to invade the wall of segments of dog femoral vein maintained in a perfusion culture system. In common with invasive variants selected in bladder organ cultures, only a few passages were needed through this tissue to isolate variant cell populations which contain a high proportion of invasive cells (Table 3). After only 4 passages, the variant population was composed almost exclusively of invasive cells, as shown by the fact that recovery and reinculatation of the "noninvasive" fraction revealed the presence of large numbers of invasive cells (Table 3).

As in the case of CAM-selected variants, the invasive behavior of bladder- and blood-vessel-selected variants (Tables 2 and 3) is highly reproducible in separate assays and remains stable in cells grown in vitro for up to 3 months or stored in liquid nitrogen for up to 6 months following initial isolation.

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### Table 1

<table>
<thead>
<tr>
<th>Passage no.</th>
<th>% of inoculated cells penetrating bladder wall</th>
<th>% of &quot;nonpenetrating&quot; cell fraction penetrating bladder wall</th>
</tr>
</thead>
<tbody>
<tr>
<td>1</td>
<td>1.3 ± 0.4</td>
<td>ND</td>
</tr>
<tr>
<td>2</td>
<td>16.4 ± 2.7</td>
<td>4.9 ± 2.4</td>
</tr>
<tr>
<td>3</td>
<td>29.7 ± 4.5</td>
<td>15 ± 2.7</td>
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<td>4</td>
<td>32.5 ± 5.2</td>
<td>23.4 ± 3.6</td>
</tr>
<tr>
<td>5</td>
<td>34.8 ± 4.3</td>
<td>29.3 ± 4.0</td>
</tr>
<tr>
<td>6</td>
<td>30.7 ± 4.2</td>
<td>32.6 ± 5.1</td>
</tr>
</tbody>
</table>

* Cell-associated $^{125}$I-IdUrd radioactivity recovered in the central well as a percentage of the total radioactivity in the original cell inoculum.

**Table 2**

Invasiveness of B16-F1 mouse melanoma cells during serial passage in bladder organ cultures from C57BL/6 mice

<table>
<thead>
<tr>
<th>Passage no.</th>
<th>% of inoculated cells penetrating bladder wall</th>
<th>% of &quot;nonpenetrating&quot; cell fraction penetrating bladder wall</th>
</tr>
</thead>
<tbody>
<tr>
<td>1</td>
<td>2.8 ± 5.7</td>
<td>ND</td>
</tr>
<tr>
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<tr>
<td>6</td>
<td>30.7 ± 4.2</td>
<td>32.6 ± 5.1</td>
</tr>
</tbody>
</table>

ND, not done.

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Invasion of Different Tissues by Variants Selected in CAM, Bladder, and Vein. Selection of cells for invasiveness in one tissue may not necessarily enable them to invade other tissues. This was demonstrated by showing that CAM-passaged invasive variants did not differ from unselected parent F1 cells in their ability to invade bladder or vein (Table 4). In contrast, bladder- and vein-passaged variants were highly invasive in the CAM, urinary bladder, and vein preparations (Table 4).

Formation of Spontaneous and Experimental Metastases by Invasive Variants. Assay of the ability of CAM-passaged invasive cell variants to form experimental metastases after i.v. injection or spontaneous metastases after s.c. injection into the external ear revealed that their metastatic activity did not differ significantly from parental B16-F1 cells (Table 5). In contrast, cells from invasive variants selected by passing in bladder organ culture (BP series) or perfused vein (BV series) produced significantly more pulmonary metastases than did parent B16-F1 cells following both i.v. (experimental metastasis) and s.c. (spontaneous metastasis) injection (Table 5). Indeed, the metastatic activity of these invasive variants is comparable to that of the highly metastatic B16-F10 line which was selected from the B16-F1 line for its ability to produce lung metastases following i.v. injection (5).

DISCUSSION

A variety of in vitro systems have been used to study invasion of normal tissues by malignant tumor cells. Attempts to study invasion by allowing normal and tumor cell populations to interact in monolayer culture (1-3, 13, 15) are probably over-simplified since tumor cells are not required to invade an organized tissue matrix. Invasion of tumor cells into aggregates of normal cells (10, 16) suffers from similar disadvantages. In addition, doubts must be expressed about the functional significance of tumor cell infiltration into aggregates in view of...
recent data showing that identical infiltrative behavior is displayed by inert particles such as Sephadex beads (42). In vitro studies on the ability of tumor cells to invade explanted tissues such as chick blastoderm (21), CAM (4, 12, 14, 24), heart (22), mesonephros (30), skin (24), wingbud (39), murine lung (35), or human decidual tissue (36) are more relevant to tumor invasion in vivo, but most of these assays do not permit accurate quantitation of invasion or recovery of the invasive cells. The assay methods reported in this paper satisfy both these requirements.

We describe here new methods for the selection of tumor cell variants with increased abilities to invade a variety of tissues in vitro. By comparing sublines selected in different tissues, information can be obtained on whether a common set of cellular properties are selected or if the properties expressed by invasive cells depends on the tissue in which the selection was made. Our results indicate that the invasive variants selected in vitro in different tissues vary significantly in their properties, despite being derived from the same parent cell line. The ability to isolate cells with different invasive phenotypes from a common parent cell population is consistent with the growing body of evidence that many malignant tumors are heterogeneous and contain subpopulations of cells with widely differing invasive and metastatic properties (for reviews, see Refs. 7 to 9 and 27).

Our results demonstrate that B16 melanoma variants selected for invasiveness in the chick CAM do not exhibit comparable invasive behavior in bladder tissue or veins. In contrast, invasive variants selected in the latter 2 tissues are highly invasive in all 3 tissues. Successful penetration of the CAM by tumor cells might be considerably less demanding since the assays do not offer a formidable mechanical barrier comparable to the bladder wall or the medial and adventitial elements of veins. Invasive variants selected by passing in bladder or vein have thus probably been subjected to a more demanding set of selection pressures than were CAM-passaged variants. This may also explain the more rapid selection (lower number of passages) of highly invasive cells in bladder and vein compared with CAM. If bladder and vein do indeed constitute more demanding selection systems than the CAM, the phenotypes that best cells for invasion in bladder and vein would be expected to be more specialized and thus probably would be present in the parent cell population at a lower frequency than the phenotypes suitable for invasion of CAM. If this interpretation is correct, variant cell populations selected in bladder and vein would from the outset be more uniform than their counterparts selected in CAM. The restricted diversity of the invasive phenotype in bladder- and vein-passaged cells would mean that cell subpopulations with highly invasive properties would quickly become dominant. In contrast, the greater heterogeneity of invasive phenotypes in CAM selected cells would mean that overgrowth and dominance of a single or a few highly invasive phenotype(s) could take much longer.

The mechanisms involved in tissue invasion by tumor cells are not known. Release of tissue destructive enzymes from tumor cells, particularly lysosomal hydrolases and collagenolytic enzymes (for reviews, see Refs. 26, 31, and 38), has been proposed as one possible mechanism. Examination of the enzyme profiles of invasive variants of the type isolated in the present study may be useful in defining what role lytic enzymes play in tissue invasion. Two reservations must be expressed, however, about attempts to correlate cellular enzymic activity with invasive potential: (a) secretion of enzymes in vitro may merely reflect the physiological potential of cells in culture and have little relevance to events in vivo; (b) the relative importance of different enzymes may not only vary between different types of tumor cells but also be influenced by the nature of the tissue(s) encountered by the tumor cells (see below). For example, enhanced production and release of plasminogen activator accompanies neoplastic transformation of a diverse range of cell types (33), and it has been speculated that this enzyme might facilitate tumor cell invasion (32, 37). Definitive evidence to support this proposal is yet to be presented. Studies in this (11) and other laboratories (23) indicate that, at least for B16 melanoma cells, increased production and secretion of plasminogen activator is not correlated with enhanced invasive or metastatic behavior.

The ability of invasive tumor cells to invade into, and escape from, blood vessels may be correlated with their ability to degrade the basement membrane of capillaries and venules (8, 17, 18, 27). The predominant form of collagen in the capillary basement membrane is type IV collagen and it is, therefore, of interest that the B16-BL6 variant described in this paper has been found to produce high levels of type IV collagenase compared with the parent B16-F10 line (19). The B16-BP8 and BV8 variants isolated in the present study also show a significant increase in collagenolytic activity compared with the F1 parent cells. CAM-passaged variants lack collagenolytic activity, but they rapidly acquire this property when passaged in either bladder or vein.

The present results indicate that variant sublines selected in vitro for increased invasiveness in bladder or vein also display a greater ability to form spontaneous metastases in vivo than did their parental cells. This suggests that the factors responsible for the isolation of cells with the invasive phenotype coincidently select for cellular properties required for the successful completion of other steps in the metastatic process. The variants selected in vein or organ cultures of bladder (BP series) are also more metastatic than are parent B16-F1 cells when injected i.v. In contrast, the variant selected in intact bladder (BL6) is less metastatic than is its parent line (B16-F10) when injected i.v. We consider that these differences in the ability of bladder-selected variants to form experimental metastases after i.v. injection results from the fact that they are derived from different parent cells. In the case of organ culture passaged variants derived from the B16-F1 line, selection for invasion results in cells with enhanced metastatic ability. Detection of the latter is facilitated, however, by the low metastatic activity of the parent cells. In contrast, the BL6 variant was selected from the F10 line which is already highly metastatic when injected i.v. (6). Formation of experimental metastases by cells injected directly into the circulation eliminates the need for primary invasion, and the outcome of metastasis in this situation may depend more on factors such as cell survival in the bloodstream, deformability, embolization, arrest, extravasation, and multiplication in organ parenchyma rather than invasive capacity. Under these conditions, it is perhaps not surprising that the parent F10 cells, selected for precisely these properties, are more successful in producing experimental metastases. However, when the BL6 variant is placed s.c.

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Selection of Invasive Variants

G. Poste, unpublished observations.
or i.m., where invasiveness is a prerequisite for metastasis, it is able to exploit its advantage over the F10 cells and metastasize more effectively.

The importance of the selection procedure in determining the invasive phenotype is also illustrated by the present results in the properties of CAM-passaged cells. These variants do not differ from parental B16-F1 cells in their ability to invade other tissue (i.e., bladder or vein). In addition, invasive variants selected in the CAM do not differ from the B16-F1 parent cells in their metastatic behavior in vivo. Comparison of CAM-selected variants, in which invasiveness and metastatic ability are functionally dissociated, with variant sublines selected in bladder and vein in which these traits are coupled could provide insight into the contribution of specific cellular alterations to these particular behavior properties.

As emphasized elsewhere (6, 27), the search for properties uniform to all invasive cells may well be unproductive. We consider, however, that the likelihood of identifying properties which are expressed with a relatively high degree of consistency in invasive tumor cells will be substantially increased by studying variant cell populations selected specifically for this phenotype.

REFERENCES

In Vitro Selection of Murine B16 Melanoma Variants with Enhanced Tissue-invasive Properties


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