Selection of Metastatic Variants from Heterogeneous Tumor Cell Lines Using the Chicken Chorioallantoic Membrane and Nude Mouse

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ABSTRACT

The chicken chorioallantoic membrane was used to select variant tumor cell subpopulations from the murine melanoma B16-BL6 and the rat glioma C6 cell lines. Tumor cells were deposited on the chicken chorioallantoic membrane of eggs 10 days postfertilization. Upon hatching, chickens were autopsied, and organs were removed, minced, and implanted s.c. in C57BL/6J mice (for melanoma) or nude mice (for glioma). A glioma growing s.c. from a chicken lung implant metastasized to the liver of the recipient nude mouse, and a melanoma growing s.c. from a chicken liver implant metastasized to the lung of its murine host. The s.c. melanoma contained distinct black and gray areas. Cell lines were established from the s.c. glioma (C6-V-1), from a metastasis of the C6-V-1 tumor (C6-V-2), and from the black and gray regions of the melanoma. Marked differences in lung colonization were seen 14 days after 1 x 10^6 parent BL6, Black, or Gray cultured cells were injected by tail vein into C57BL mice. In four separate experiments, fewer than 15 lung foci per mouse were found when BL6 cells were injected, whereas 100 to several hundred lung melanoma colonies per mouse were observed when Black or Gray cells were inoculated. Four of 18 nude mice bearing the s.c. C6-V-1 glioma developed liver metastases; no metastases have been observed in 15 nude mice bearing the s.c. parent C6 glioma. Significant differences in sensitivities to antineoplastic drugs were demonstrated between parent and variant glioma cell lines. The 33-fold increase in sensitivity to vincristine determined for C6-V-1 cells compared to the vast majority of cells in the metastatic potential from primary human tumors and human tumor cell lines. Some of these results have been presented in preliminary form (43).

INTRODUCTION

There is now compelling evidence that many animal and human tumors are heterogeneous for multiple phenotypic characteristics (5, 7, 10, 22, 25). Single neoplasms have been documented to contain subpopulations that are morphologically heterogeneous (8, 9, 41), antigenically distinct (29, 37, 39), or which differ in elaboration of specific cell products (markers) (1, 20). Karyotypic heterogeneity has been reported in a number of animal and human cancers (8, 9, 30, 41, 47). As might be expected, neoplastic subpopulations within individual tumors also have been shown to be heterogeneous for invasive or metastatic ability (11, 27, 32, 38). Only a relatively small proportion of cancer cells within a tumor appears to possess the functional specialization required to complete all the steps in the metastatic process (5, 22, 44). Since most patients die of metastases, these are the cells that are of major concern clinically. The demonstration by several groups that cells from metastases can have different sensitivities to antineoplastic drugs, compared to cells in the primary tumor, further complicates the task of the clinician (4, 17, 24, 28, 42, 45).

The ability to select, isolate, and study human neoplastic cells that have high metastatic potential will be of great importance in our efforts to improve treatment of metastatic disease in cancer patients. We have developed a methodology for selecting and isolating cells from tumor lines which are more invasive or metastatic when compared to the vast majority of cells in the neoplasm. This method, using the CAM in situ, followed by outgrowth of micrometastatic foci in nude (or syngeneic) mice, has been utilized successfully for C6 rat glioma and the B16 mouse melanoma. The methodology and the characterization of the selected variant lines are described in this report. This technique has direct applicability for the selection of cells with metastatic potential from primary human tumors and human tumor cell lines. Some of these results have been presented in preliminary form (43).

MATERIALS AND METHODS

Cell Cultures. C6 rat glioma cells were obtained from the American Tissue Culture Collection (Rockville, Md.). The line was originally established from an N-nitosomethylurea-induced rat glial tumor by Benda et al. (3). Parent and variant cells were cultured in Ham's F-10 growth medium supplemented with 15% heat-inactivated horse serum, 2.5% heat-inactivated fetal calf serum, 0.15% sodium bicarbonate, 100 units of penicillin per ml, 100 ^g of streptomycin per ml, 2.5 ^g of Fungizone per ml (all from GIBCO), and 20 ^g of gentamicin per ml (Schering Corp., Kenilworth, N. J.).

The B16-BL6 murine melanoma was obtained from Mason Research Institute (Worcester, Mass.). The culture was maintained in Eagle's minimum essential medium with Earle's salts (GIBCO). The culture was supplemented with 10% heat-inactivated fetal calf serum, minimum essential medium vitamin solution (GIBCO), 0.58 mg L-glutamine per ml, 0.1 mm nonessential amino acids, 0.1% sodium pyruvate, 0.21% sodium bicarbonate, 0.4 ^m 4-(2-hydroxyethyl)-1-piperazineethanesulfonic acid, and the same antibiotic concentrations that were used for the glioma medium.

All cell lines were maintained at 37° in a humidified atmosphere of 5% CO_2 in air. The culture medium was replaced with fresh medium at 3- to 4-day intervals after plating. For routine passage, cells were exposed to 0.25% trypsin and replated in dishes (Falcon No. 3001 or 3002, Falcon Plastics, Oxnard, Calif.) or flasks (Falcon No. 3013).
Inoculation of Cells onto the CAM. SPF-Utility fertile hen eggs for experiments were obtained from SPAFAS (Norwich, Conn.). They were kept in an incubator at 37°C with 60% relative humidity, and were turned twice daily to prevent adhesion of the chorioallantoic membrane. Eggs 10 days postfertilization were used as recipients of tumor cells. Each egg was candled and a Y-shaped junction of blood vessels in the CAM was marked on the shell with a pencil. The egg shell was cleaned with 70% alcohol and a 25-sq-mm hole was drilled through the shell. The shell membrane was then excised with a pair of fine-pointed forceps.

C6 or B16-BL6 cells in culture were trypsinized, and the cell suspensions were centrifuged (5 min at 1400 rpm in a Sorvall GLC-1 centrifuge). The pellet was washed twice with sterile 0.9% NaCl solution, resuspended in 0.9% NaCl solution, and approximately 3 x 10^6 cells in 0.1 ml were applied onto the CAM through the window.

The opening was sealed with sterile Parafilm (American Can Co., Greenwich, Conn.), and eggs were incubated 10 days to hatching. On Day 20 eggs were opened and chicken embryos were sacrificed and autopsied. Lungs and livers were dissected, minced, and transplanted s.c. in nude mice for glioma cells, and in C57BL/6J mice for melanoma cells. Sections of CAM, CAM-bearing tumor, and chicken organs were sectioned and fixed for histology.

Establishment of Variant Cell Lines. The s.c. tumors were removed, placed in approximately 0.5 ml of medium, and minced. Three to 5 pieces from the minced preparation were placed in a 35-mm plastic culture dish. An additional 1 ml of medium was added to the dish both before and after 1 hr of incubation. Medium was replaced with fresh medium every 2 to 3 days. Explants showing no cellular outgrowth by 5 to 7 days were removed by suction. The explant pieces containing viable cells grew out within 1 to 2 days and produced confluent monolayers within 1 to 2 weeks for both cell lines.

A C57BL mouse implanted s.c. with chicken liver fragments was found to have a neoplasm consisting of black and gray regions. Each of the 2 regions was treated separately, and cell cultures were established from the heavily pigmented and less pigmented zones of the tumor.

A nude mouse bearing a s.c. glioma (called C6-V-1) was found to have metastases to the liver. The liver was minced and treated with a digest of 2.5% trypsin. The resulting single-cell suspension was plated in 60-mm tissue culture dishes (Falcon). After 2 weeks, colonies of glioma cells could be seen among the attached liver cells. Passage of the primary culture resulted in cultures that contained rat glioma (C6-V-2) cells without murine liver or connective tissue cell contamination.

Characterization of Cell Lines. The growth properties of the glioma and melanoma cell lines were determined according to the methods previously reported from our laboratory (6-9). These methods are summarized briefly here.

For determination of cell-doubling times, replicate culture dishes received inocula of 1 x 10^5 (glioma) or 5 x 10^6 (melanoma) cells, and cultures from 2 dishes were harvested separately each day and counted with a hemacytometer. Cell numbers versus days in culture were plotted on semilogarithmic paper, and doubling times were calculated from the curves.

Plating efficiencies in plastic tissue culture dishes were obtained from the results of experiments performed to determine doubling times. The percentage of attached cells was calculated from the number of cells plated on Day 1 and the number of cells attached on Day 2. To determine adherence to glass, 1 x 10^6 glioma cells were plated on tissue culture chamber slides (Niles Laboratories, Inc., Naperville, Ill.) and examined for attachment after 24 hr of incubation.

Saturation densities were calculated from the data from the experiments done to determine cell-doubling times. The number of cells present in confluent 35-mm (Falcon) dishes provided the maximum cell number per sq cm.

For growth in semisolid medium, harvested cells were resuspended in 0.5% agar (Difco Laboratories, Detroit, Mich.) in complete growth medium. One ml of this suspension was layered onto a 2-ml base of 1.0% agar in medium in a 35-mm Falcon culture dish. Cloning efficiencies in agar were determined in inoculum by statistical counting, in duplicate dishes under a light microscope, of colonies in 20 to 30 separate fields containing more than 30 cells. Agar dishes were given 0.5 ml of complete medium twice a week for 2 weeks, and colonies were enumerated on Day 14.

For chromosome analysis, exponentially growing cultures were incubated with 0.2 μg of Colcemid per ml (GIBCO) for 5 to 6 hr. Cells were harvested and chromosome preparations were made, using standard techniques. The slide preparations were stained with Giemsa, and 50 metaphases were examined for each cell line using a light microscope (8).

In Vitro Drug Sensitivity Studies. Replicate tissue culture dishes were inoculated with 1 x 10^6 glioma cells in Ham's F-10 growth medium on Day 1. Cells were harvested from 3 of the dishes on Day 2 and counted with a hemacytometer to determine the number of cells per dish at the time of drug addition. Varying concentrations of drugs were added to the remaining cultures on Day 2. Drug-treated and control cultures were harvested and counted on Day 5, and the number of doublings for untreated cells was calculated from the cell numbers in control dishes on Days 2 and 5. The molar drug concentration inhibiting the number of doublings in the 72 hr period by 50% was calculated from the dose-response curve in which cell numbers were plotted against molar drug concentrations (6).

In Vivo Studies. Athymic, nude mice bearing the nu/nu genotype on an outbred Swiss background are bred and maintained in the Roger Williams Cancer Center Animal Care Facility. Nude mice, 6 to 8 weeks of age, were used as hosts for injections of glioma cell lines. One million cells were injected s.c. into the flank region. Specific-pathogen-free C57BL/6J black mice were obtained from Animal Resources (The Jackson Laboratory, Bar Harbor, Maine) and maintained in our Animal Care Facility. Mice 4 to 8 weeks of age were used as hosts for injections of melanoma cell lines; 1 x 10^6 cells were injected s.c. Tumors were measured twice weekly, and mice were autopsied and examined for metastases at 14 days or upon death.

In the lung colonization experiments, melanoma cultures were harvested, and the cells were resuspended in 0.9% NaCl solution to give the desired concentration of viable cells. A 0.2-ml inoculum of the single-cell suspension (1 x 10^6 cells) was injected into the tail vein of C57BL mice matched for age and sex. Mice were autopsied after 14 days, and lungs were inflated with 1.5 ml distilled water and fixed in 10% neutral buffered formalin. Visible colonies of melanoma cells on the surfaces of the lungs were counted independently by 2 observers using a dissecting microscope.

Histology. Tissues taken for light microscope examination were fixed in 10% neutral buffered formalin and embedded in paraffin. Sections (4 μm), cut on a standard microtome, were mounted on glass slides and were stained with hematoxylin and eosin.

RESULTS

C6 Rat Glioma

Selection of Variant Lines. C6 cells deposited on the CAMs of five 10-day fertilized hens' eggs developed into solid tumors upon hatching of the chickens 10 days later. Histological sections of the CAM tumor showed an appearance typical of high-grade astrocytoma or glioblastoma multiforme. For the most part, the tumor was composed of spindle-shaped or "spongioblastic" cells with fibrillar cytoplasmic processes and large oval nuclei. The cells were arranged in interweaving fascicles, as well as less regular, looser clusters; pseudopalisades of cells surrounded large zones of necrosis. Occasional polygonal and oval cells were present with more pleomorphic nuclei, and mitoses were prevalent. The histological appearance was identical to that of tumors produced by s.c. inoculation of nude mice with C6 cells (Figs. 1 and 2).
Chickens from eggs with tumor-bearing CAMs were sacrificed and autopsied at hatching. Organs were excised and minced, and representative portions of liver and lung were either fixed for histology or were implanted s.c. into nude mice. Histopathological examination of the organ sections did not reveal micrometastases. From the 5 eggs studied, one small nodule developed after a 6-week latency period from a chicken lung fragment implanted into an athymic host. The nodule was removed and minced, and portions were fixed for histology, explanted into tissue culture dishes, or were passaged s.c. into several nude mice. The host mouse was autopsied, and numerous tumor foci were seen in the liver which were identified histologically as metastatic glioma. The histological patterns of these metastases and of the tumor nodules growing out of the s.c. chicken lung transplant were similar to each other and to the C6 tumors produced by inoculating cultured C6 cells into CAMs or nude mice. The s.tumor that developed from the implanted lung tissue had the additional appearance of endothelial proliferation and perivascular fibrosis, both within the tumor and in the surrounding fat and connective tissue. These findings are common in glial tumors (40) (Fig. 3). The liver metastases in nude mice consisted of stellate clusters of polygonal, oval, and fusiform cells similar to those in the CAM and s.c. tumors, associated with fairly extensive necrosis and inflammation, endothelial proliferation in central veins and sinusoids, and dysplastic, reactive changes in the surrounding hepatocytes (Fig. 4).

A primary cell culture was established from the explanted tumor pieces and was designated C6-V-1. Cells were readily passaged into other plastic dishes, and cultured C6-V-1 cells (1 x 10⁶ inoculum) injected s.c. into nude mice readily produced tumors. Glioma tissue fragments also produced tumors in recipient nude mice, and the C6-V-1 line has been propagated continuously in vivo, as well as in vitro. Liver metastases were also found in 3 of 18 other mice on separate occasions during routine passage of the C6-V-1 tumor line. The liver from one of these mice was minced, trypsinized, and a single-cell suspension was plated in tissue culture dishes. Glioma cell colonies grew out in the dishes, resulting in the establishment of the C6-V-2 line. C6-V-2 cells (1 x 10⁶ cell inoculum) also produced tumors upon s.c. injection into nude mice. No metastasis to liver has ever been observed in 15 animals bearing s.c. parent C6 tumors.

Growth Characteristics of Cultured Glioma Lines. The variant C6-V-1 and C6-V-2 cells were morphologically distinct from parent C6 cells. The variant lines, both similar to one another, formed parallel chains of cells which stretched out in a radial pattern. This radial pattern was not nearly as pronounced in the parent cell line. The growth properties of the C6, C6-V-1, and C6-V-2 cell lines are shown in Table 1. The variant lines had significantly longer doubling times (31 to 32 hr) than the parent line (21 hr). Plating efficiencies on plastic and cloning efficiencies in agar were reduced for C6-V-1 and C6-V-2 cells compared to parent C6 cells. In early passages, C6-V-1 cells did not adhere at all to glass surfaces, whereas C6 cells had a plating efficiency of 68% for 1 x 10⁶ cells placed on glass culture slides. There was no significant difference in modal chromosome number and range among the 3 lines.

Drug Sensitivities. The chemosensitivities of the 3 cell lines to several drugs used clinically in the treatment of brain cancer were determined. The results are summarized in Table 2. The sensitivities of C6-V-1 and C6-V-2 cells to each agent differed markedly from the values of the molar dose of drug that inhibited the number of doublings by 50% determined for parent C6 cells. The 33-fold increase in sensitivity to vincristine determined for C6-V-1 cells compared to C6 cells is striking. In all cases, the variant cell lines were more sensitive to each drug tested than was the parent line, illustrating once again that cells from a heterogeneous tumor line selected for metastatic or invasive potential can also differ in their responses to chemotherapeutic drugs, compared to the parent cells. As can be seen from Table 2, C6-V-1 and C6-V-2 cells also differ from one another as well as from the parent C6 cells in their sensitivities to each of the 4 drugs tested.

B16-BL6 Murine Melanoma

Selection of Variant Lines. BL6 cells deposited on the CAM of ten 10-day fertilized eggs had produced a solid tumor by the time the chickens had hatched. Chickens were sacrificed and autopsied upon hatching. Organs were minced, and selected tissues were fixed for histopathological examination, which showed no evidence for metastasis. C57BL mice were inoculated s.c. with liver and lung fragments. A large tumor developed after an 8-week latency period in a mouse that had received a chicken liver implant from one of the 10 eggs studied. The tumor was variable in color and contained a black (highly pigmented) region, and a gray (less pigmented) region. Small fragments from each zone were explanted into tissue culture dishes or were examined histologically, and other pieces were implanted s.c. into syngeneic mice. The host mouse was autopsied and the metastatic foci were seen in the lungs. The morphological appearances of the tumor produced by injection of BL6 cells into mice, the BL6 tumor on the CAM, the “black” region of the tumor growing out from the chicken liver implant, and the mouse lung metastases were similar.

Histologically the CAM tumor was composed of large, polygonal, epithelioid cells with abundant, vacuolated cytoplasm and indistinct cytoplasmic borders. Most of the cells contained a large amount of fine to coarsely granular melanin pigment. The nuclei were large and oval with a vesicular appearance, coarsely clumped chromatin, and large nucleoli. There were numerous mitoses. The cells grew in sheets and clusters among large, blood-filled spaces and delicate capillaries, such that most of the tumor was composed of RBC (Fig. 5). The s.c. tumor that developed from chick liver implanted in the C57BL mouse was considerably more cellular than the CAM tumor, having a fine vascular stroma of capillaries without large, blood-filled spaces. The cells resembled those of the CAM tumor, but notably fewer contained melanin. Approximately one-fourth
while the gray region was virtually amelanotic. The gray region of the cells in the black region of the tumor contained melanin, to produce melanotic tumors, whereas neoplasms produced from the expiant cultures. These cells produced melanomas upon injections into C57BL mice, and the 2 variants could thus be propagated in vivo as well. The Black line in the mouse continued to produce melanotic tumors, whereas neoplasms produced by implanting Gray tumor fragments contained gray, black, and sometimes white (completely amelanotic) regions.

Growth Characteristics of Melanoma Lines. The morphologies of cultured Black and Gray cells were quite similar, but differed somewhat from the appearance of the parent line. The variant cells were somewhat more spindle shaped and often developed longer processes, compared to BL6 cells. The monolayer cultures of the variant lines showed a much less orderly, more haphazard arrangement of cells than was observed with BL6 cultures. The doubling times, clonogenicities in agar, plating efficiencies on plastic, and saturation densities of the Black, Gray, and BL6 lines were quite similar.

Assessment of Metastatic Potential. Tail vein (i.v.) injection of cultured melanoma cells was used to determine the abilities of the 3 lines to colonize the lungs of syngeneic mice (Table 3). The results were somewhat variable among experiments, but the Black and Gray lines consistently showed a markedly increased capability of forming tumors in the lung, compared to BL6 cells under the conditions of this study. Fewer than 15 lung foci per animal were observed with an inoculum of 1 x 10^6 BL6 cells in 4 independent experiments, whereas approximately 10^2 to several hundred lung melanoma colonies were enumerated per animal when 1 x 10^6 Black or Gray cells were used. A dose response was also obtained in experiments in which 1 x 10^5 and 2.5 x 10^5 tumor cells were injected.

DISCUSSION

A tumor weighing 1 g contains approximately one billion cells. However, patients seldom present with thousands or even hundreds of metastases (5, 22). Clearly, only a few cells in a tumor are capable of completing the metastatic process. Furthermore, the cells responsible for the secondary tumor deposits can differ in their chemosensitivities, compared to cells in the primary tumor (4, 17, 24, 28, 42, 45). Thus, not only do a select few cells in a cancer possess the functional specialization to complete the many steps involved in dissemination but these cells may also respond differently to treatment protocols, compared to the great majority of (nonmetastatic) cells in the primary tumor. These findings present the clinician with a problem of quite some magnitude. It will be necessary to study the cells in human cancers that have a propensity for metastasis in order to develop protocols effective against these cells. However, before one can study such variant cells, they must be selected, isolated, and recovered. The CAM outgrowth model described here provides one means of obtaining human cancer cells with more aggressive properties.

Both the CAM and the nude mouse have been used as tools to study the growth and metastasis of human cancers. Murphy demonstrated, almost 70 years ago, that a human tumor could be transplanted successfully to the CAM (31). Armstrong et al. (2) recently reported that invasion of cultured murine and human tumor cells across the intact CAM was quite limited, whereas invasion across a traumatized CAM was extensive. Vogel and Berry (48) grew a number of human brain tumors on CAM surfaces, and some of these passed serially to other CAMs. Gitterman et al. (19) grew human adenocarcinoma, epidermoid carcinoma, and sarcoma cells on CAMs. Furthermore, they transplanted embryo lungs from eggs with HEP-3-bearing CAMs onto new CAM surfaces and observed tumor development from the lung implants. Ossowsky and Reich (35) obtained similar findings with HEP-3 cells; tumors developed from chicken embryo lung fragments transplanted to naive CAMs following an initial CAM inoculation with these human cancer cells. None of these investigators reported establishing cell lines from the tumors growing out of the chicken lungs. The HEP-3 line would seem to be unusually aggressive for a human cancer line, as the

Table 2
Sensitivities of glioma cell lines to anticancer drugs

<table>
<thead>
<tr>
<th>Cell line</th>
<th>Vincristine</th>
<th>VP-16</th>
<th>VM-26</th>
<th>Vinblastine</th>
</tr>
</thead>
<tbody>
<tr>
<td>C6</td>
<td>1.9 ± 0.5 x 10^-8</td>
<td>1.7 ± 0.35 x 10^-4</td>
<td>3.3 ± 0.28 x 10^-4</td>
<td>8.9 ± 4.21 x 10^-4</td>
</tr>
<tr>
<td>C6-V-1</td>
<td>5.8 ± 2.5 x 10^-10</td>
<td>3.69 ± 1.6 x 10^-9</td>
<td>1.6 ± 0.55 x 10^-9</td>
<td>8.13 ± 3.84 x 10^-10</td>
</tr>
<tr>
<td>V6-V-2</td>
<td>4.2 ± 1.3 x 10^-10</td>
<td>1.7 ± 0.3 x 10^-8</td>
<td>2.3 ± 0.54 x 10^-8</td>
<td>3.47 ± 0.22 x 10^-10</td>
</tr>
</tbody>
</table>

*ID_50, molar dose of drug that inhibited the number of doublings by 50%; ID_50 = 0.22 x 10^-10.*

Table 3
Lung-colonizing abilities of BL6 and variant lines

<table>
<thead>
<tr>
<th>Experiment</th>
<th>Lung colonies</th>
</tr>
</thead>
<tbody>
<tr>
<td>BL6</td>
<td>Black</td>
</tr>
<tr>
<td>1 (5a)</td>
<td>8 ± 2</td>
</tr>
<tr>
<td>2 (5)</td>
<td>4 ± 1</td>
</tr>
<tr>
<td>3 (6)</td>
<td>14 ± 7</td>
</tr>
<tr>
<td>3 (6)</td>
<td>135 ± 43</td>
</tr>
<tr>
<td>4 (5)</td>
<td>11 ± 4</td>
</tr>
<tr>
<td>4 (8)</td>
<td>77 ± 25</td>
</tr>
</tbody>
</table>

*Numbers in parentheses, number of mice.

Mean ± S.E.
tumors from organ implants appeared after only 8 to 10 days (the limit of the technique, as CAMs are inoculated 10 days postfertilization, and chickens hatch 10 days later). It is our conclusion that an outgrowth method should provide a time frame of several months to permit microdeposits of human tumors to develop into visible cancers, as many human tumors most likely will need a longer period of time to become macroscopic. Our results suggest that the nude mouse would be appropriate for such a strategy.

It has been generally believed that human tumors metastasize only rarely in nude mice, although a few cases of dissemination in athymic hosts have been described (18, 28, 46). The recent report by Kyriazis et al. (26) indicates that human tumor metastasis in nude mice may occur more commonly than has been appreciated. This result is quite encouraging but must be substantiated in many laboratories in order to be generally useful. Hanna and Fidler (21) have used 3-week-old nude mice as hosts for tail vein inoculations of mouse and rat tumor cells; lung colonization occurred with both lines. Tsuruo and Fidler (45) subsequently used this method to obtain lung colonies following i.v. injection of human melanoma A-375 cells. Their approach will undoubtedly be useful for in vivo selection of human cancer cells with increased metastatic potential, but it may not be suitable for general use for 2 reasons. First, the success of this approach is predicated on the use of young nude mice maintained under strict pathogen-free conditions necessary to prevent the early appearance of natural killer cells, which are felt to be responsible for the failure of xenograft tumor cells to metastasize in nude mice (21, 45). Many laboratories do not have the facilities required to produce and maintain pathogen-free young athymic mice. Second, the tail vein injection technique bypasses several steps required for a spontaneous metastasis. This lung colonization assay is quite useful to assess the experimental metastatic potential of a clone, and we have used this assay in our evaluation of the Black and Gray lines. However, there is also a need for selection methods that require aggressive cells to complete more, or all, of the steps in the metastatic process. This technique described here provides investigators with a method that does not require young pathogen-free nude mice, and also allows detection of metastases produced under in vivo conditions that perhaps more closely resemble spontaneous dissemination, compared to the lung colonization method.

The lines established in this study from micrometastases in chicken organs differ in several important respects from the parent cell line originally deposited on the CAM. The morphologies of the variant lines are distinct from the parent lines. C6-V-1 and C6-V-2 cells have different growth rates, plating efficiencies on plastic, and cloning efficiencies in soft agar compared to C6 cells. The increased drug sensitivities of the C6-V-1 and C6-V-2 cells compared to C6 parent cells are marked. Especially striking is the 33-fold increase determined for vincristine with C6-V-1 cells, and the 26-fold increase measured for vinblastine with C6-V-2 cells. These data are in agreement with results from other laboratories, showing that cells from metastases can have significantly different chemo sensitivities compared to cells from the parent tumor or cell line (4, 17, 24, 28, 42, 45).

The differences among parent and variant cell lines for growth properties or drug sensitivities for each tumor system are important in documenting the heterogeneity of the parent cell line, and in validating that selection has been achieved. For the model described here to be useful in selecting more aggressive subpopulations of tumor cells, however, it is important to demonstrate differences between parent and variants with respect to metastatic potential. In 4 cases we observed metastasis of the C6-V-1 line growing s.c. in nude mice to the livers of these animals. By contrast, metastasis of the s.c. parent C6 tumor to the liver of athymic mice has never occurred. Our experiments with i.v. injections of BL6, Black, and Gray cells indicate that the 2 variant lines have a much greater ability to colonize lungs than do BL6 cells. Based on these results, we conclude that the variant lines selected by our methodology have a greater metastatic potential than do the parent cells deposited on the CAM.

The progression of tumors with time to become more autonomous and more aggressive was described in the classical studies of Foulds (12–16). Nowell (33, 34) has hypothesized convincingly that most tumors are monoclonal at their inception, but that variants are generated due to the genetic instability of cancer cells. Some of these clones are eliminated due to extrac- and intratumor selection pressures; other “more favored” variants survive and proliferate. Fould’s theory of tumor progression and Nowell’s explanation for it have provided the basic description of the evolution of intratumor heterogeneity and have anticipated the increasing attention recently accorded intraneoplastic diversity. The extension of past and recent efforts in this field will depend on our ability to obtain for study, cells from both human and animal neoplasms that are functionally specialized for aggressive behavior. We have reported here the synthesis of 2 distinct methodologies, the CAM technique and the use of the nude mouse, to provide a system suitable for the selection and isolation of human and animal cancer cells with increased metastatic potential. This model should prove useful in future studies of the functional heterogeneity existing within individual human tumors.

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REFERENCES

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Fig. 1. Section of tumor grown from C6 cells deposited on CAM of 10-day fertilized hen’s egg. Spindle-shaped cells growing in fascicular pattern and palisading around a zone of necrosis (top) are typical of high-grade astrocytoma or glioblastoma multiforme. H & E, × 50.

Fig. 2. Higher magnification of CAM tumor showing the characteristic fibrillar cytoplasmic processes and dense oval and fusiform nuclei of the neoplastic giant cells. H & E, × 160.

Fig. 3. Section of tumor that developed after s.c. implantation of chick lung tissue in nude mouse. Note similarity to CAM tumor with the additional appearance of endothelial hyperplasia and perivascular fibrosis (top center) common in glial tumors. H & E, × 63.

Fig. 4. Focus of glial metastatic to liver of same mouse with s.c. tumor. Note histological similarity to CAM and s.c. tumors. Hepatocytes show reactive nuclear changes (top right corner). H & E, × 100.

Fig. 5. Section of tumor grown from BL6 cells deposited on CAM of 10-day fertilized hen’s egg. Melanoma cells have large, oval, vesicular nuclei and abundant cytoplasm with fine and coarse melanin pigment (arrows). Nucleated RBC, with uniform, small, dense nuclei, fill irregular vascular spaces among clusters of melanoma cells. H & E, × 160.

Fig. 6. Black region of melanoma that developed in C57BL mouse after s.c. implantation of chick liver tissue. Melanin is present (arrow), although seen in fewer cells than in the CAM tumor. H & E, × 160.

Fig. 7. Gray region of same s.c. tumor. Melanoma cells are morphologically similar but contain no melanin. H & E, × 160.

Fig. 8. Melanoma metastatic to lung of same mouse with s.c. tumor showing histological similarity to the original CAM tumor. H & E, × 160.
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