Selective Events in the Metastatic Process Defined by Analysis of the Sequential Dissemination of Subpopulations of a Mouse Mammary Tumor

Cheryl J. Aslakson and Fred R. Miller

ABSTRACT

To identify selective steps in metastasis, those that eliminate nonmetastatic tumor cells more efficiently than metastatic cells, we have evaluated the sequential dissemination of tumor cells from a mammary fatpad, using both metastatic (4T1 and 66cl4) and nonmetastatic (67NR, 168FARN, and 4TO7) subpopulations of a single mouse mammary tumor. Each of these variant subpopulations is resistant to one or more selective drugs so they could be quantitatively identified by colony formation in selective media. We found that the 2 metastatic cell lines metastasized by different routes and that the nonmetastatic tumor cell lines failed at different points in dissemination. Line 67NR did not leave the primary site; clonogenic tumor cells were not detected in the nodes, blood, or lungs during the experiment (7 weeks). Tumor line 168FARN disseminated from the primary tumor because clonogenic cells were cultured from the draining lymph nodes throughout the experiment. However, dissemination essentially stopped in the node as cells were rarely isolated from blood, lungs, or livers. Whether 168FARN cells failed to reach these tissues or were killed very rapidly after traversing the lymph node is unknown. Line 4TO7 cells disseminated via the blood and were consistently recovered from lungs by day 19 but failed to proliferate. This panel of 5 subpopulations thus identifies different points of selective failure in tumor cell dissemination and should be valuable in the assessment of antimetastatic therapies.

INTRODUCTION

The metastatic process is a sequence of steps (invasion, intravasation, transport, arrest, extravasation, and growth) that must be accomplished by cancer cells before distant metastases are established (1, 2). Metastasis of even highly metastatic cells is normally a very inefficient process; most tumor cells that enter the bloodstream do not develop into metastatic nodules (3, 4). Both random and selective events may be responsible for this metastatic inefficiency (5). Metastasis is selective because: (a) cells isolated from spontaneous metastases may be more metastatic than the original parent tumor (6); and (b) genetically stable metastatic and nonmetastatic sublines have been characterized (6-8). Nonmetastatic cell lines are unable to complete one or more steps in the metastatic cascade, whereas metastatic cell lines must be able to complete all steps involved in the metastatic process (2). Although random events in the metastatic process eliminate the majority of tumor cells irrespective of metastatic phenotype (9), one can define any step that more efficiently eliminates cells of nonmetastatic lines than metastatic lines as a selective step. If host immune functions are important for the elimination of potentially metastatic cells, the selective events may be the best targets for therapeutic intervention with biological response modifiers.

MATERIALS AND METHODS

Mice. Female BALB/c mice, 8-12 weeks old, were produced in our animal colony from a BALB/c breeding colony established by cesarean derivation of a litter of mice from BALB/cF1 mice obtained from the Cancer Research Laboratory, Berkeley, CA. The Michigan Cancer Foundation Animal Facility is fully accredited by the American Association for Accreditation of Laboratory Animal Care.

Tumor Cell Lines. Tumor subpopulation lines 66, 67, 168, and 410.4 were isolated from a single spontaneously arising mammary tumor from a BALB/cF1 mouse (8, 13). Line 66cl4 is a thioguanine-, ouabain-resistant variant of line 66 (14). Line 168FARN is a diamino-purine-, genetin-resistant variant clone of line 168 obtained by transfecting the bacterial pSV2 plasmid containing the neomycin resistance gene into 168FARN (15). Similarly, the genetin-resistant 67NR was obtained by transfecting line 67. Line 4TO7 is a thioguanine-, ouabain-resistant variant of 410.4 (16). Line 4TO7 is a thioguanine-resistant variant that was selected from 410.4 without mutagen treatment. Line 4TO7 spontaneously metastasizes to both the lung and liver as evidenced by the formation of visible nodules in these organs in mice bearing line 4TO7 primary tumors. Line 66cl4 spontaneously metastasizes to the lung. Lines 67NR, 168FARN, and 4TO7 are highly tumorigenic but very rarely metastasize spontaneously.

Media. Cells were grown in DMEM-10, Dulbecco’s modified Eagle’s medium supplemented with 5% fetal calf serum, 5% newborn calf serum, 1 mM mixed nonessential amino acids, 2 mM L-glutamine, penicillin (100 units/ml), and streptomycin (100 μg/ml). To prepare cells for injection, flasks were rinsed with 0.25% trypsin in 0.05% EDTA, washed once, and suspended in DMEM-10.

Detection of Dissemination. Tumor cells were suspended to a final concentration of 1 x 10^7 cells/ml in DMEM-10. Mice were anesthetized with sodium pentobarbital (65 mg/kg body weight), and 1 x 10^4 tumor cells in 0.01 ml were injected into a no. 4 mammary fatpad (17). At various times, groups of 4-5 mice were anesthetized and bled from the right ventricle with a syringe rinsed with heparin (6.29 mg/10 ml). Lungs were minced into 1-mm^3 pieces and presoaked for 60 min in 5 ml of an enzyme cocktail containing 1 mg/ml collagenase type IV (Sigma Chemical Company, St. Louis, MO) and 36 units of elastase (ICN Biomedicals, Inc., Costa Mesa, CA) at 4°C. The samples were mechanically dispersed with 4 sequential 30-s and 3 sequential 1-min periods in a Stomacher blender (Tekmar Company, Cincinnati, OH). Following each dispersion period,

Received 8/8/91; accepted 12/30/91.

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1 Supported by USPHS Grant CA28366 from the National Cancer Institute and a grant from the Concern Foundation.

2 To whom requests for reprints should be addressed, at: Michigan Cancer Foundation, 110 East Warren Avenue, Detroit, MI 48201.

3 The abbreviations used are: DME-10, supplemented Dulbecco’s modified Eagle’s medium; lUrld, iododeoxyuridine.
a portion of the cell suspension was removed and an equal volume of DME containing 10% calf serum (DME-10) added (10).

Livers were minced and an enzyme solution containing collagenase type I (Sigma) and hyaluronidase (Sigma), each at a concentration of 1 mg/ml, in a final volume of 5 ml was added. After a 15-min incubation at 37°C, 5 ml of DME-10 were added and samples were mechanically dispersed for 5 sequential 1-min periods. Between bursts, a portion of the cell suspension was removed and an equivalent volume of DME-10 added (10).

All cell suspensions were plated in DME-10 containing the appropriate selective drug and incubated for 10 to 14 days in 10% CO₂-air atmosphere at 37°C. The colonies were fixed with Carnoy’s solution, stained with crystal violet, and counted, and total colony-forming cells per organ calculated.

RESULTS

Sequential dissemination of tumor cells from a mammary fatpad was evaluated using both metastatic (4T1 and 66cl4; Fig. 1) and nonmetastatic (67NR, 168FARN, and 4TO7; Fig. 2) subpopulations of a mouse mammary tumor. Line 4T1 appeared to metastasize primarily, but not exclusively, by a hematogenous route because clonogenic tumor cells were recovered significantly more frequently (P < 0.001, χ² analysis) from the blood (38 of 58 mice) than from draining lymph nodes (12 of 58 mice). Figure 1A depicts 1 of 2 experiments in which the sequential spread of 4T1 from a mammary fatpad was monitored. Lungs were seeded early (clonogenic 4T1 cells were first detected at day 7, and 5 of 5 mice had clonogenic 4T1 cells in the lung by day 14) and the lung tumor burden increased on subsequent assay days with an apparent doubling time of 30 h. (The apparent doubling time is an underestimate because it not only measures the growth rate of occult metastases but also reflects continuous seeding of tumor cells from the primary tumor.) Livers were seeded later, in that 4T1 cells were first detected at day 21 and incidence did not reach 100% until day 28. Seven mice were necropsied at day 31; all had visible lung nodules (43 ± 7; mean ± SEM) and 5 had visible liver nodules (2.0 ± 0.8). In a second experiment, lungs were again seeded early (66% incidence by day 9) and the lung tumor burden increased on subsequent assay days. Clonogenic 4T1 cells could be detected in the liver within days after detection in the lungs. Ten mice from the second experiment were sacrificed for necropsy between days 34 and 40. All 10 had visible nodules on the surface of the lungs (49 ± 6.5) and 5 mice had a visible nodule on the surface of the liver.

Clonogenic tumor cells were recovered from both the blood (27 of 82 mice) and lymph nodes (31 of 59 mice) of mice bearing line 66cl4 tumors in a mammary gland. χ² analysis suggests that line 4T1 more often metastasized hematogenously than did line 66cl4 (P < 0.02) and that line 66cl4 more often metastasized via the draining lymph node than did line 4T1 (P < 0.01). Fig. 1B depicts 1 of 2 experiments in which the sequential spread of 66cl4 from a mammary fatpad was monitored. Clonogenic cells were detected in lungs of 66cl4 tumor bearing mice on day 14 and all mice had detectable tumor cells in the lungs by day 28. After day 28, expansion of the tumor cell population in the lung proceeded at an exponential rate with an apparent doubling time of 39 h. In addition, clonogenic cells were recovered from livers on day 35 in this experiment, but all 8 mice sacrificed for necropsy at day 50 had visible nodules in the lung (26 ± 3) but not liver nodules. Metastatic nodules have never been observed in livers of mice with 66cl4 primary tumors. In a second experiment with 66cl4, clonogenic cells were again detected in lungs on day 14, and the apparent doubling time in the lungs after day 28 was 36 h. Seven mice from this experiment were sacrificed for necropsy at day 50. All 7 had visible nodules in the lungs (6 ± 2); no liver nodules were found.

The nonmetastatic line 67NR appeared to be unable to intravasate. Clonogenic cells were not recovered from any of the tissues (blood, lymph nodes, lungs, or liver) sampled from a total of 44 mice over a period of 7 weeks for 2 experiments, with one exception in which 3 clonogenic cells were recovered from a draining lymph node of one animal on day 7 in one experiment (Fig. 24). Livers were dissociated and plated in selective media in one experiment (no clonogenic cells were detected) but not in the other. None of the mice sacrificed at days 49–50 for necropsy in the 2 experiments had visible metastases or detectable clonogenic cells in any organ. The size...
Fig. 2. Recovery of clonogenic tumor cells from mice bearing nonmetastatic primary tumors. A, mice were given injections of 1 × 10⁶ 67NR tumor cells in the mammary fatpad. At multiple intervals, groups of mice were bled and then sacrificed, and lymph nodes and lungs removed. B, mice were given injections of 1 × 10⁷ 168FARN tumor cells in the mammary fatpad. At multiple intervals, groups of mice were bled and then sacrificed, and lymph nodes and lungs were removed. The draining lymph node in the mammary fatpad was removed through day 21, after which the size of primary tumor made it difficult to find the lymph node. The draining brachial lymph nodes were collected throughout the experiment except on day 50. C, mice were given injections of 1 × 10⁵ 4TO7 tumor cells in the mammary fatpad. At multiple intervals, groups of mice were bled and then sacrificed, and lymph nodes, lungs, and livers removed. The draining lymph node in the mammary fatpad was removed through day 19, after which the size of the primary tumor made it difficult to find the lymph node. The draining brachial lymph node were collected throughout the experiment. The tissues were processed as detailed in “Materials and Methods.” The data were expressed as geometric means for each tissue. The symbols indicate which tissues were sampled and the day on which sampling was done: •, all tissues for that day; *, blood and lymph nodes only.

of the primary 67NR tumors at the time of necropsy (6.6 ± 1.5 g and 11.2 ± 4.6 g in the 2 experiments) far exceeded the size of either 66cl4 (2.6 ± 0.3 g and 2.3 ± 0.8 g at necropsy) or 4T1 (1.9 ± 0.2 g and 1.7 ± 0.5 g at necropsy) primary tumors, when metastatic nodules were detectable in animals with the latter 2 tumor lines.

The nonmetastatic line 168FARN cells spread through the lymphatics, since clonogenic cells were recovered from draining lymph nodes in the mammary gland (Fig. 2B). In a second experiment, low numbers of clonogenic 168FARN cells were occasionally recovered from lungs (1 animal of 6 at necropsy on day 51 had 43 clonogenic cells) and livers (none of the 6 animals at necropsy had detectable cells in the liver, but 2 of 5 mice on day 21 had 13 and 65 clonogenic cells, and 2 of 5 mice on day 28 had 8 and 15 clonogenic cells in the liver). Although clonogenic 168FARN cells could be recovered from draining lymph nodes, clonogenic cells were not recovered in either experiment from the blood of any of the 61 mice bearing line 168FARN tumors. Visible nodules were not observed on lungs at necropsy, and clonogenic cells were recovered in only one animal as noted above despite the presence of very large primary tumors (9.3 ± 1.1 g and 8.6 ± 2.2 g at necropsy for the 2 experiments).

The nonmetastatic line 4TO7 appeared to be able to complete all steps of metastasis except the final one. Line 4TO7 spread via the blood to lungs and occasionally to livers, but did not establish progressively growing metastatic nodules. Fig. 2C depicts one of 2 experiments in which the sequential spread of 4TO7 from a mammary fatpad was monitored. Clonogenic cells were recovered from the lungs at day 19. At necropsy, visible nodules were absent but clonogenic 4TO7 cells were recovered from lungs in 4 of 5 mice (primary tumor weight of 1.35 ± 0.32 g on day 28) with a geometric mean of 32 clonogenic cells detected.

In a second experiment with 4TO7, clonogenic cells were recovered from the lungs on day 7 after tumor cells were
implanted into a mammary fatpad. The discrepancy between
the two 4TO7 experiments is most likely due to the fact that
the primary 4TO7 tumors appeared earlier in the second ex-
periment and, thus, tumors were larger on the various assay
days. In the second experiment, necropsies were done on day
38 when primary tumor weights were 2.85 ± 0.42 g. Visible
nodules were absent, but clonogenic 4TO7 cells were recovered
from lungs of all 8 mice with a geometric mean of 1086
clonogenic cells. However, if primary 4TO7 tumors are sur-
gically removed, clonogenic cells disappear from the lung within
1 week (data not shown), indicating that the presence of clon-
ogenic 4TO7 cells in the lungs is dependent upon continuous
seeding from primary tumors rather than on proliferation of
tumor cells in the lung.

The growth properties of all 5 subpopulations in monolayer
culture in vitro were similar. The population doubling times
ranged from 16 h for 67NR and 66cl4 to 21 h for 168FARN.
The clonogenic potential (no. of colonies formed/no. of cells
plated) ranged from 0.54 for 4T1 and 4TO7 to 0.68 for 67NR.

The addition of heparinized blood had little effect on the
clonogenic potential of the subpopulations. As compared to
clonogenic potential in medium alone, recovery in the presence
of heparinized blood ranged from 86% for 168FARN to 97%
for 67NR.

Line 67NR cells (100 cells) were plated with lymph node
cells (2 × 10⁶) to determine if the failure to detect 67NR in
draining lymph nodes was due to lymph node cell mediated
suppression of colony formation by 67NR in vitro. Neither
lymph node cells from normal mice (0.63 clonogenic potential)
nor lymph node cells from mice bearing 67NR tumors (0.57
clonogenic potential) markedly affected growth of 67NR in
vitro.

Clonogenic potential in selective media was not reduced by
the addition of 1 × 10⁷ lung cells in a 100-mm dish. In fact,
when fewer than 10 clonogenic tumor cells were mixed with 1
× 10⁷ lung cells, more tumor cell colonies were recovered than
in controls without added lung cells. Thus, the sensitivity of the
method for detecting occult clonogenic tumor cells seems to be
limited only by the ability to recover tumor cells during the
tissue dissociation procedure.

The relative resistance of the 5 subpopulations to the method
used to dissociate lungs was determined (Fig. 3). Line
168FARN was the most sensitive and 4TO7 was the least
sensitive. However, the data indicate only a 2-fold differential
recovery between these extremes. It is not likely that this
differential sensitivity to organ disruption was responsible for
the differential recovery of sublines from the lung.

DISCUSSION

It is not uncommon for tumor sublines to display heteroge-
nous metastatic potential. Mouse melanomas (19, 20), lympho-
mas (21, 22), mammary carcinomas (8, 23, 24), lung carcinomas
(25, 26), a fibrosarcoma (27), a colon carcinoma (28), rat
mammary carcinoma (29), rat prostate carcinoma (30), human
renal cell carcinoma (31), and human melanoma (32) have all
been used to derive sublines with high and low metastatic
potential. Further characterization of such subpopulations fre-
quently uncovers differences in phenotypes of apparent impor-
tance in the metastatic sequence such as the production of
enzymes for degradation of extracellular matrix and basement
membrane (33–36), reduced production of tissue inhibition of
metalloproteinases (37), migration (38–42), response to auto-
motility factor (43), laminin expression (40, 44), laminin bind-
ing (45), invasion in vitro (46, 47), lectin expression (48),
hyomorph (49) and heterotypic aggregation (50), anchorage
independence (51), expression of g-protein (52), gap junctions
(53), response to growth factors (54, 55), antigenicity (56, 57),
and differential susceptibility to tumoricidal macrophages (58),
natural killer cells (59, 60), or T-cells (61, 62).

It may not be prudent to assume that properties expressed in
vitro, despite their correlation with the end result, i.e., formation
of visible metastatic nodules, reflect similar properties mechan-
istically responsible for selection in vivo during the course of
metastasis. Thus, for example, a metastatic line that produces
some proteolytic enzyme and a nonmetastatic line that does not
produce that enzyme might invade equally well but differ
in their ability to respond to some growth factor or have a
differential sensitivity to immune resistance in the involved
organ. On the other hand, 2 cell lines, 1 metastatic and 1
nonmetastatic, may both produce some proteolytic enzyme that
enables both cell lines to invade, but the nonmetastatic line fails
to survive some subsequent step. Thus, one can conclude
neither that a property is important in metastasis because it correlates
with metastasis nor that a property is unimportant because it
does not correlate with metastasis. Analysis of specific steps in
the metastatic cascade would advance the understanding of the
basic biology of metastasis and allow a more critical evaluation
of the roles of the many factors implicated in metastasis.
Furthermore, if host immune functions are important for the
elimination of potentially metastatic cells, then it may be the
selective events that are likely to be the best targets for therapeu-
tic intervention. Random events in the metastatic process
eliminate the vast majority of tumor cells irrespective of meta-
static phenotype. Weiss (9) argues that, if 99.9% of cells of a
metastatic line injected i.v. fail to form metastatic nodules (e.g.,
100 colonies are formed after injecting 10⁵ cells) and 99.999%
of cells of a nonmetastatic line injected i.v. fail to form meta-
static nodules (e.g., 1 colony is formed after injecting 10⁵
cells), the metastatic process is essentially random. Although
this may be true on a statistical basis, each individual metastasis
may have profound clinical consequences. Weiss (9) suggests
that “if any population of cancer cells entering the metastatic
cascade goes sequentially through five randomly traumatic steps
associated with invasion, etc., each of which kills 90% of the
cells, then only 0.001% of the initial cellular input from the
primary cancer will form metastases.” However, to further
develop Weiss’s example, if we assume that, in addition to the
random trauma that eliminates cells of both metastatic and
nonmetastatic lines equally, the occurrence of a selective pro-
cess at any of the steps accounts for an additional log kill of the
nonmetastatic cells, the occurrence of 2 selective steps in the
cascade would decrease the likelihood of developing metastatic
disease by 100-fold. Although these calculations of log kills are
hypothetical, they illustrate the impact that even weak selective
events might have on the final outcome in an otherwise random
metastatic process. To date, knowledge of which steps in the
metastatic cascade are selective has been limited by deficiencies
in existing methodology to analyze quantitatively the clonogen-
icity of potentially metastatic cells at various points in the
metastatic cascade.

Bioassays have been used to estimate the number of tumor
cells with replicative potential present in tissues of tumor-
bearing experimental animals, but the methods were insensitive
and not quantitative (63–65). By mixing lethally irradiated cells

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with blood or minced lungs, Mayhew and Glaves (66) were able to increase the sensitivity of their bioassay for B16 melanoma or Lewis lung carcinoma cells, but a threshold of 10–100 cells still existed. Another undesirable feature of a bioassay is the requirement for multiple animals to test for the presence of metastatic tumor cells in 1 experimental tumor-bearing animal.

Susuki (67) enzymatically dissociated lungs with protease type IX and then plated the cell suspensions from tumor-bearing mice onto irradiated feeder layers. After 2 weeks, colonies were fixed, stained, and counted (67, 68). In our own studies, the background lawn of normal tissue cells, even without using a feeder layer, made it impossible to quantitate colonies formed by some of our tumor cell lines that do not form dense colonies. Furthermore, we have occasionally observed distinct colonies in wells of cell suspensions prepared from the lungs of normal mice when plated in nonselective media. By using tumor cells with drug resistance markers, it is possible to simultaneously quantitate and unambiguously identify the tumor cell colonies developing in the presence of selective media (10–12, 18, 69, 70). Since first demonstrating our ability to follow the sequential spread of mammary tumor cells from a mammary fatpad (18), we have significantly improved our methods for dissociating the lung and liver so that greater than 50% of the tumor cells (identified by [125I]IUrd labeling) are recovered (10).

All 5 mouse mammary tumor subpopulations used in these experiments colonize the lung if a sufficient number of cells are injected i.v. However, only 2 are metastatic based on their ability to metastasize spontaneously from a mammary fatpad; the other 3 obviously are unable to complete some step in the metastatic process. We found a significant difference in the route of dissemination by the metastatic subpopulations 66cl4 and 4T1. Because venolymphatic anastomoses may allow tumor cells to move readily from one compartment to the other (blood/lymphatics) (71), one might expect that lymph node metastases act as a generalizing site for blood-borne metastases. This may be the case for subpopulation 66cl4, which was detected in the blood of 33% of tumor-bearing mice and in the lymph nodes of 53%. However, subpopulation 4T1 appears to enter the blood stream (66% of mice) without lymph node involvement [21% of mice (P < 0.001)]. Our results with these 2 subpopulations are consistent with those of Alterman et al. (72) and Weiss and Ward (73, 74). Although bloods from tumor-bearing mice were not evaluated, Alterman et al. (72) concluded that 2 metastatic clones of the B16 melanoma must metastasize hematogenously to the lung because cells were recovered from the lung by bioassay before they could be detected in regional draining lymph nodes. Both B16 melanoma (72) and Lewis lung carcinoma (73) appear to metastasize independently via both lymphogenous and hematogenous routes. Weiss and Ward (74) also found that the rat mammary carcinoma MT-100-TC metastasized by both lymphogenous and hematogenous routes, but hematogenous transport was apparently due to entry of cells into blood vessels within the primary tumor rather than via venolymphatic anastomoses because tumor cells were rarely found in blood vessels associated with regional lymph nodes.

Previous studies have compared different tumor models and subpopulations to elucidate mechanisms of metastatic inefficiency. Clones of K1735 murine melanoma were able to colonize lungs in syngeneic euthymic mice, able to colonize lungs in athymic but not euthymic mice, or unable to colonize lungs in either athymic or euthymic mice (75). The authors classified these clones as metastatic, metastatic antigenic, and nonmetastatic, respectively. Aggregation and initial arrest of [125I]IUrd-labeled cells was similar for metastatic and nonmetastatic clones, but clearance within 24 h of arrest was much more rapid for a nonmetastatic clone than for a metastatic clone (75). Similarly, we have described that arrest was similar, but 3 of our subpopulation panel had different rates of clearance postarrest (11); 168FARN was cleared more rapidly than 66cl4 or 4T07.

Death of arrested cells, as reflected by [125I]IUrd clearance rates, is not the only postarrest selective event in metastasis. Clearance rates do not necessarily correlate with lung colony forming efficiency of tumor cells injected i.v. Reeve and Twemlyman (76) found that the more metastatic of 2 murine sarcoma clones was more rapidly killed (faster clearance of labeled cells), but the clonogenic population expanded more rapidly in the lungs for the highly metastatic clone than for the poorly metastatic clone. If injected i.v., our nonmetastatic subpopulation 4T07 is better able to colonize the lungs than the metastatic line 66cl4 or the nonmetastatic line 168FARN, and these 3 subpopulations expand in the lung in the same rank order of 4T07 > 66cl4 > 168FARN (11). Because 4T07 does reach the lungs from primary tumors growing in mammary fatpads but does not form metastatic nodules, perhaps it should be classified as metastatic antigenic (75). However, in the study of Aukerman et al. (75), line 4T07 would have been classified as metastatic rather than metastatic antigenic because the criterion used for classification was lung colony formation following i.v. injection rather than spontaneous metastasis.

Intravasation appears to be an important selective step in metastasis. Our line 67NR appears unable to leave the primary site. Glaves (77), comparing B16 melanoma and Lewis lung carcinoma, and Weiss et al. (78), comparing B16 melanoma variants, found that cells more highly efficient at colonizing lungs following i.v. injection were similar in abilities to spontaneously metastasize from i.m. tumors. Bioassays for tumorigenic cells in the bloods from tumor-bearing mice indicated that low colony-forming efficiency lines compensated by releasing more tumorigenic cells into the blood.

By defining the selective step in metastasis at which our nonmetastatic subpopulations fail, we have begun to establish an experimental model that will allow the validation of many phenotypes implicated in metastasis. A battery of subpopulations with defined deficiencies will facilitate the verification of the role in metastasis played by a given cell characteristic. This panel of subpopulations also has obvious value in the analysis of tumor cell interactions. Various pairs have been used to demonstrate clonal dominance (16, 79) and interactions affecting responses to chemotherapeutic drugs (80, 81). In our studies, clonal dominance does not correlate with metastatic phenotype as has been reported by Kerbel et al. (82) and Waghorne et al. (83). In addition, nonmetastatic subpopulations derived from the mouse mammary tumor used in our studies may metastasize in the presence of some metastatic subpopulations (84). This phenomenon has also been observed by Waghorne et al. (83). One reason for developing drug-resistant metastatic and nonmetastatic lines with defined metastatic deficiencies is to investigate mechanisms by which cell interactions alter metastatic phenotypes. With the methodology described, we continue to isolate and characterize new variants for these purposes.
## SELECTION OF METASTASIS

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**ACknowledgments**

We thank Dr. G. H. Heppner for many helpful discussions, D. McEachern and J. Bukowski for expert technical assistance, and M. Peterson for preparation of this manuscript.

**References**


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