# Metastasis Results from Preexisting Variant Cells Within a Malignant Tumor

Abstract. *Clones derived in vitro from a parent culture of murine malignant melanoma cells varied greatly in their ability to produce metastatic colonies in the lungs upon intravenous inoculation into syngeneic mice. This suggests that the parent tumor is heterogeneous and that highly metastatic tumor cell variants preexist in the parental population.*

The question of why cancer cells metastasize is one of the most important issues in tumor biology. In human cancer it is the process of metastasis, the formation of secondary tumor foci at distant sites, that eventually defeats the efforts of both surgeon and clinical oncologist. In spite of the importance of this phenomenon, little is known about the pathogenesis of metastatic foci or their relationship to the primary tumor. Studies with transplantable tumors in rodents have shown that both host factors and properties of the tumor cells can contribute to the success or failure of the metastatic process (*1*).

Earlier studies with the B16 melanoma in syngeneic C57BL mice showed that the majority of tumor cells injected intravenously die very rapidly in the circulation, and only about 0.1 percent survive and yield metastases (*2*). Further experiments suggested that the survival of these few tumor cells was not a random occurrence, but was due to certain unique properties of the surviving cells (*3*). In this study, we wished to determine whether these unique metastatic cells preexisted in the tumor cell population, or whether they arose during metastasis by a process of adaptation to local environmental conditions. If highly metastatic variant cells could be shown to preexist in the parent population, this would support the suggestion by Nowell (*4*) that tumor cell variants arise within developing tumors, are subjected to host selection pressures, and are responsible for the emergence of new sublines with increased malignant potential.

To distinguish between these possibilities, we performed an experiment similar in design to the classical fluctuation test devised by Luria and Delbrück to distinguish between selection and adaptation in the origin of bacterial mutants (*5*). In our study a cell suspension of the B16 melanoma parent line was divided into two parts. One portion was used to inject syngeneic C57BL/6 mice intravenously. The other portion was used to produce clones, which were then also injected intravenously into groups of C57BL/6 mice (Fig. 1). Eighteen days after the tumor cells were injected, the number of lung metastases in each recipient was counted. If the number of metastatic foci in the lungs of the mice receiving the cloned sublines was similar to the number of foci seen in mice receiving the parent line, this would indicate that the parent population was homogeneous and that the metastatic foci probably resulted from adaptation during the process of metastasis. Alternatively, if the cloned sublines gave rise to widely different numbers of lung colonies, this would suggest that the parent tumor was heterogeneous and that cells of both high and low metastatic potential preexisted in the parent population.

This experiment was performed with the transplantable B16 melanoma, which originated spontaneously in a C57BL/6 mouse in 1954. We obtained it from Jackson Laboratory, Bar Harbor, Maine, in 1970 and passaged it several times in syngeneic recipients prior to establishing it in cell culture (*6*). After four to five passages in vitro, this parent line was frozen and stored in liquid nitrogen until used in these studies. The cells were thawed and cultured for three passages in vitro to obtain the starting material for this experiment. Seventeen single cell clones were derived by a combination of the soft agarose and microculture techniques (*7*). All cell suspensions for intravenous injection were prepared in an identical manner by light trypsinization from cultures in exponential growth

Table 1. Metastases resulting from intravenous injection of cells from parent B16 tumor line and its in vitro cloned sublines.

| Source of B16 | Number of mice per group | Number of pulmonary tumor colonies per mouse* | Number of pulmonary tumor colonies: Median | Number of pulmonary tumor colonies: Range | Number of animals with extrapulmonary metastases |
|---|---|---|---|---|---|
| Parent line | 60 | 8, 20, 24, 26, 27, 27, 27, 28, 28, 30, 30, 30, 30, 31, 31, 32, 33, 33, 33, 34, 34, 35, 36, 36, 37, 37, 39, 40, 40, 40, 41, 43, 44, 44, 44, 44, 44, 46, 46, 46, 49, 56, 56, 59, 60, 62, 64, 66, 66, 67, 68, 69, 72, 72, 72, 73, 78, 84, 98, 131 | 40.5 | 8–131 | 8/60 ovary, 6/60 liver, 3/60 gut, 11/60 lymph nodes, 2/60 adrenal, 1/60 heart, 4/60 kidney, 1/60 nasal sinuses |
| Clone 16 | 10 | 2, 2, 2, 3, 3, 4, 6, 9, 11, 15 | 3.5 | 0–15 | 0/10 |
| Clone 15 | 11 | 2, 3, 3, 4, 5, 5, 6, 8, 9, 15, 20 | 5 | 2–20 | 1/11 lymph node |
| Clone 12 | 9 | 0, 0, 1, 4, 6, 10, 16, 27, 34 | 6 | 0–34 | 0/9 |
| Clone 24 | 9 | 5, 6, 7, 9, 10, 11, 13, 23, 29 | 10 | 5–29 | 1/9 ovary, 1/9 liver, 1/9 lymph node |
| Clone 19 | 10 | 0, 3, 4, 8, 9, 17, 20, 22, 23, 42 | 13 | 0–42 | 0/10 |
| Clone 7 | 10 | 0, 5, 9, 10, 16, 18, 19, 27, 29, 43 | 17 | 0–43 | 0/10 |
| Clone 21 | 8 | 1, 5, 8, 15, 21, 22, 23, 48 | 18 | 1–48 | 1/8 lymph node |
| Clone 18 | 11 | 0, 1, 1, 2, 8, 36, 41, 42, 70, 75, 91 | 36 | 0–91 | 0/11 |
| Clone 5 | 10 | 2, 25, 31, 44, 45, 46, 67, 74, 78, 171 | 45.5 | 2–171 | 0/10 |
| Clone 6 | 9 | 5, 41, 42, 89, 99, 115, 115, 149, 232 | 99 | 5–232 | 0/9 |
| Clone 17 | 9 | 104, 110, 132, 144, 150, 151, 173, 206, 210 | 150 | 104–210 | 0/9 |
| Clone 3 | 9 | 160, 166, 196, 208, 214, 229, 241, 261, 450 | 214 | 160–450 | 1/9 lymph node |
| Clone 1 | 9 | 73, 114, 153, 165, 237, 272, 273, 290, 321 | 237 | 73–321 | 0/9 |
| Clone 2 | 10 | 7, 28, 206, 218, 241, 268, 336, 353, 378, 450 | 254.5 | 7–450 | 0/10 |
| Clone 13 | 9 | 50, 62, 79, 91, 260, 306, 320, 338, 350 | 260 | 50–350 | 2/9 ovary, 1/9 liver |
| Clone 14 | 9 | All nine >500 | >500 | | 2/9 ovary |
| Clone 9 | 10 | All ten >500 | >500 | | 2/10 adrenal, 1/10 kidney, 1/10 brain, 6/10 lymph node, 2/10 liver |

*Mice were injected intravenously with 50,000 viable tumor cells and killed 18 days later.



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phase (3). The cells were washed in culture medium, resuspended in physiologic saline, and pipetted gently to dissociate any cell clumps. Only cell suspensions of more than 95 percent viability, as measured by trypan-blue exclusion, and that were free of cell aggregates were used for injection. C57BL/6 mice were injected in the lateral tail vein with $5 \times 10^4$ viable tumor cells in a volume of 0.2 ml. Eighteen days after injection, the mice were killed. The number of pulmonary tumor colonies in each animal was counted in double-blind fashion under a dissecting microscope by two independent observers. The B16 melanoma, which appears as superficial black nodules, grows preferentially in the lungs after intravenous injection (3). Complete autopsies were performed, and all suspected extrapulmonary metastases were confirmed by microscopic examination of fixed histological sections.

Table 1 lists the number of pulmonary nodules per mouse obtained from intravenous injection of the parent B16 line and each of the 17 clones. The metastatic potential of the clones, as seen from the median number of pulmonary colonies, differs dramatically from that of the parent B16 line. In fact, only two clones, 18 and 5, are indistinguishable from the parent line, based on the Mann-Whitney U test (8). There was also considerable variation among the clones in the number and sites of extrapulmonary metastases. We conclude, therefore, that the cells with a high metastatic potential are present within the parent B16 line prior to their injection into animals.

However, the variability among the clones could have resulted from the process of cloning rather than from heterogeneity of the parent tumor. To test whether the cloning procedure could be responsible for generating these variants, clones 21 and 24 were recloned to produce several subclones. If the cloning procedure were responsible for introducing the variation, then the subclones should also exhibit wide variation in metastatic potential. The distributions of the number of lung colonies produced by the groups of subclones do not differ statistically from each other or from their respective parent clones (Table 2). This suggests that the process of cloning is not the major factor responsible for the variability of the clones seen in Table 1. We conclude that the parent B16 tumor is extremely heterogeneous with respect

Table 2. Pulmonary metastases resulting from intravenous injection of cells from B16 clones 21 and 24 and their subclones.

| B16 source | Number of mice injected | Number of pulmonary tumor colonies | |
|---|---|---|---|
| | | Median | Range |
| Parent clone 21 | 8 | 18 | 1–48* |
| Subclone 21-d | 9 | 6 | 0–45 |
| 21-b | 10 | 24 | 0–135 |
| 21-c | 10 | 38.5 | 0–118 |
| 21-a | 10 | 42.5 | 0–138 |
| Parent clone 24 | 9 | 10 | 5–29* |
| Subclone 24-b | 10 | 3.5 | 0–30 |
| 24-a | 10 | 8 | 0–48 |
| 24-c | 9 | 9 | 0–96 |

*No statistically significant differences were detected between parent clones and their subclones or among the related subclones using a two-tailed Mann-Whitney U test (8).

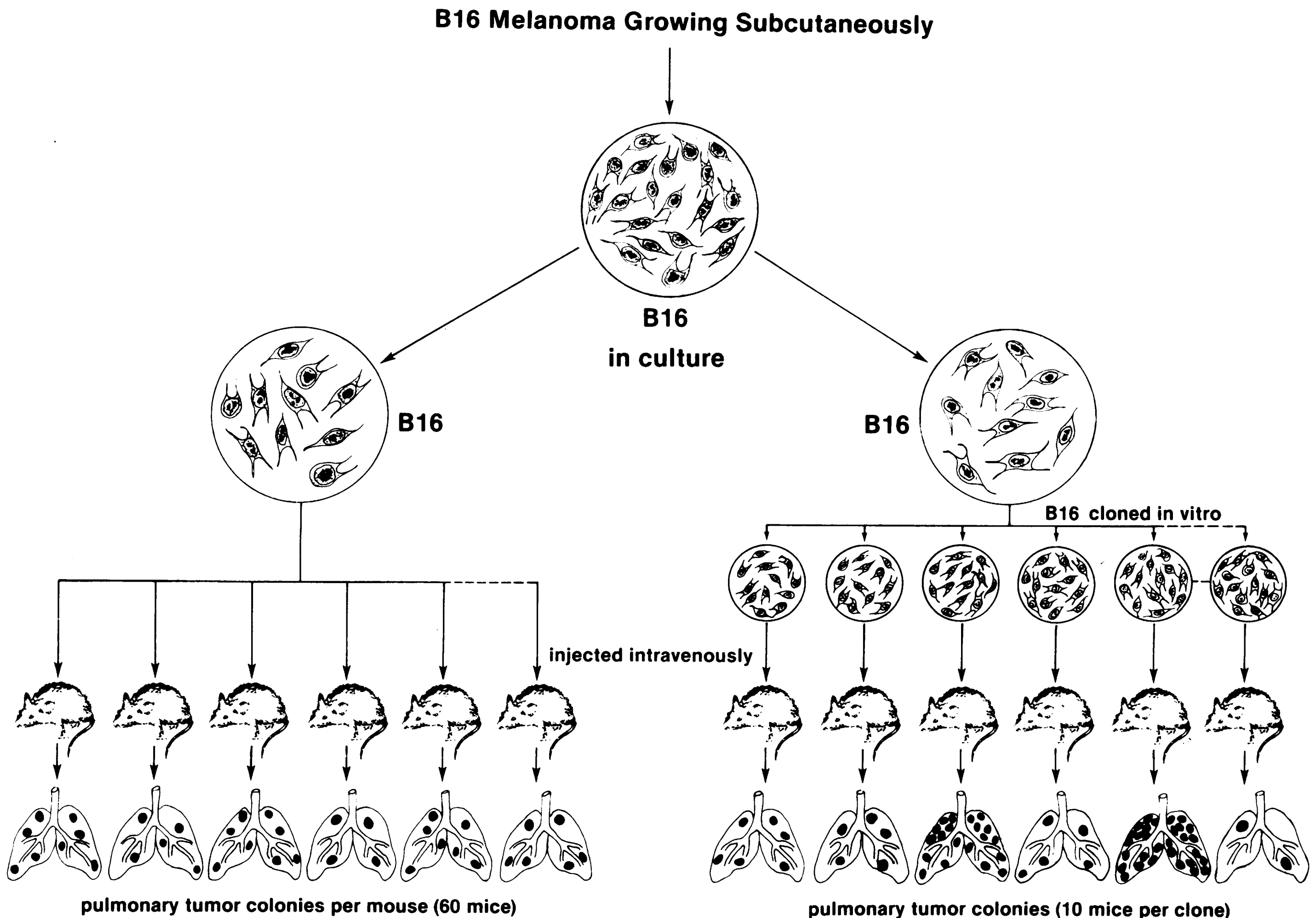


Fig. 1. Scheme for demonstrating that metastatic variants preexist within a malignant tumor. B16 melanoma grown in vitro was divided into two parts. One part was injected intravenously into syngeneic C57BL/6 mice and the other was used to produce several clones. Once established, the clones were also injected intravenously into syngeneic mice. All tumor cell suspensions were prepared in an identical manner. Mice were injected intravenously with $5 \times 10^4$ viable tumor cells and were killed 18 days later. The number of pulmonary and extrapulmonary metastases in each mouse was determined with the use of a dissecting microscope.

to the metastatic potential of its individual cells. This high degree of heterogeneity is probably attributable to the fact that the B16 melanoma has existed as a transplanted tumor for more than 20 years. It is quite likely that many variants would arise during this period by the process of mutation and selection and by epigenetic mechanisms (4). In addition, the process of metastasis is a complex one with many sequential steps. It begins with the invasion of tissues and vessels by cells originating in the primary cancer. After their entry into the circulation, most cells are arrested in the first capillary bed encountered, but some continue and are trapped in other organs. After this arrest, the tumor cells must invade the parenchyma, proliferate, establish a vascular supply, and escape host defense mechanisms in order to develop into secondary foci. A cell that acquires an increased ability to survive any one of these steps would be viewed as having an increased metastatic potential. Thus, there are probably many different pathways by which a cell could acquire an increased or decreased capacity to form a new colony at a distant site.

The possible existence of highly metastatic variant cells within a primary tumor may have important consequences for cancer therapy. Efforts to design effective therapeutic agents and procedures should be directed toward the few, albeit fatal, metastatic subpopulations. Continuing efforts to eradicate the bulk of neoplastic cells, without regard to their biological behavior, are likely to be unproductive. Perhaps the highly metastatic clones described in our study would be useful tools for testing new therapeutic approaches to cancer.

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**References and Notes**

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6. Cells were grown on plastic in Eagle's minimum essential medium supplemented with 10 percent fetal calf serum, vitamin solution, sodium pyruvate, nonessential amino acids, penicillin-streptomycin, and L-glutamine, designated complete minimum essential medium (CMEM) (Flow Laboratories, Rockville, Md.).
7. A 1 percent mixture of agarose was prepared in distilled, deionized sterile $H_2O$. The mixture was boiled and agitated, then cooled to 37°C and mixed with equal volume of double strength CMEM. A layer of CMEM and agarose was placed in a petri dish (120 by 20 mm) and the remaining mixture was kept warm at 37°C. A single cell suspension of the B16 melanoma in CMEM was adjusted to contain 2000 viable cells per milliliter. The cell suspension was diluted with an equal amount of the agarose and CMEM mixture. One milliliter of the final suspension (1000 cell/ml) was added to each petri dish, and the mixture was allowed to harden. The dishes were incubated at 37°C (5 percent $CO_2$) overnight. After that time, the dishes were examined under an inverted microscope and the positions of *isolated single* cells were noted and marked. Single cells were removed with a Pasteur pipette and placed in a Microtest II well (Falcon Plastics). Twelve hours later the wells were examined, and those with an attached single cell were identified. Tumor colonies resulting from these single cells were propagated and serially transferred to vessels of increasing size.
8. S. Siegel, *Nonparametric Statistics for the Behavioral Sciences* (McGraw-Hill, New York, 1956), pp. 116–126.
9. We thank Z. Barnes and J. Connor for technical assistance. Supported by National Cancer Institute contract N01-C0-25423 with Litton Bionetics, Inc.

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## Phi Bodies: Peroxidatic Particles that Produce Crystalloidal Cellular Inclusions

Abstract. *Unique, spindle-shaped particles (ϕ bodies) and rods with peroxidatic activity are found in certain epithelial cells of normal mice, clofibrate-fed rats, and in leukemic leukocytes. The ellipsoidal shape of ϕ bodies apparently results from the deformation of spherical granules by extrusion of axial crystalloid that subsequently fragments into rods.*

While studying the abundant peroxisomes in mouse salivary gland distal duct luminal epithelial cells with light microscopic histocytochemical methods for the direct demonstration of their isocitrate dehydrogenase (1) and catalase, we observed (2) enzyme-reactive rods as well as filaments of different dimensions (Fig. 1). Study of the size and distribution of these rods and filaments suggested that they could not be microorganisms (3). This was confirmed by correlative studies with the electron microscope, which also demonstrated that these structures were crystalloid rather than crystalline.

In our earliest studies of these rods we observed spindle-shaped particles (Figs. 1 to 3) which, at first, were thought to consist of microbodies superimposed on rods. At this time, neither the change in conformation of the peroxisome from spheroidal to ellipsoidal nor the increase in size that accompanies its transformation to the ϕ body had been recognized. Indeed, the elucidation of the ϕ body as a unique and distinct cellular particle visible with the light microscope in histocytochemical enzyme preparations was delayed by our ability to resolve several of these putative spindle-shaped particles into rods superimposed on peroxisomes with the light microscope.

Study of the specimen shown in Fig. 1, however, suggested that a considerably higher incidence of these particles than might be expected from the fortuity of superimposition could be found in certain areas of the ductal epithelium. Further study of this specimen revealed that the centers of almost all of these unique spindle-shaped particles, which could not be resolved with the light microscope as being due to overlapping peroxisomes and rods, were ellipsoidal rather than spherical. It was also noted at this time that the rods or filaments which extruded from the poles of the ϕ body invariably appeared to coincide with the major axis of its ellipsoidal center. The ϕ body centers were also generally larger than nearby spheroidal microbodies.

These observations led to the hypothesis that the deformation of the spherical microbody to an ellipsoidal shape and its increase in size could result from internal pressure exerted by crystalloid growing in the centers of the relatively small numbers of transforming microbodies prior to and during the extrusion of the same material from their poles. This proposed mechanism is similar to the "tent-pole" effect proposed for the protein crystalloid of immature eosinophil granules by which their spherical contours become angulated (4). A similar effect appears to account for the population of ellipsoidal azurophilic granules of normal neutrophilic promyelocytes which have an axial band of crystalloid (5).

Several other considerations also tended to relate ϕ bodies to free rods observed in the luminal epithelial cells of the distal ducts of mouse salivary glands. Light microscopic study of the submandibular glands of many strains of mice and several strains of rats revealed that, although nonparticulate catalase and peroxisomes were prominent in the distal salivary gland duct cells of all strains, ϕ bodies were observed only in the tissues of the rodent strains that contained catalase-positive rods or filaments and vice versa (3). This similarity in distribution, although a very important consideration, was perhaps not as significant as the frequent observation with the light