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A Murine Model of Experimental Metastasis to Bone and Bone Marrow[1]

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ABSTRACT

Bone is a common site of metastasis in human cancer. A major impediment to understanding the pathogenesis of bone metastasis has been the lack of an appropriate animal model. In this paper, we describe an animal model in which B16 melanoma cells injected in the left cardiac ventricle reproducibly colonize specific sites of the skeletal system of mice. Injection of 10^5 cells resulted in melanotic tumor colonies in most organs, including the skeletal system. Injection of 10^4 or fewer cells resulted in experimental metastasis almost entirely restricted to the skeletal system and ovary. In contrast, i.v. injection of 10^5 cells resulted in tumor colonies in the lung only. Left cardiac injection of 10^2 cells caused bone colonization, but the same number of cells injected i.v. did not colonize the lung. The number of bones with tumor colonies increased with increasing number of cells injected. Melanotic tumor colonies in the bone were characteristically distributed in the metaphysis of long bones and in the periphery of flat bones. Most animals developed paraplegia due to spinal cord compression by bony metastasis to the spine. Tumor colonization of bone occurred only in regions of bone containing hematopoietic bone marrow. This suggests that the injected tumor cells lodge, survive in the hematopoietic bone marrow environment, and grow to destroy adjacent bone. This experimental model of metastasis to bone will facilitate future studies of the pathophysiology and treatment of bone and bone marrow metastasis.

INTRODUCTION

Bone metastasis is clinically important but has been studied little in animal models (for review, see Ref. 1). In human cancer, bone is the third most common site of metastasis, and in autopsies as many as 60% of cancer patients are found to have bone metastasis (2). Also, metastasis to bone and bone marrow is most often the first clinically detected site of metastasis in breast cancer, prostate cancer, small cell lung carcinoma, and neuroblastoma (3). Metastases in bone are found invariably in sites adjacent to red bone marrow (4). This suggests that tumor cells lodge, survive, and grow in the hematopoietic bone marrow space and expand to destroy adjacent bone. Microenvironmental differences between bone marrow and other tissues (see, for example, Refs. 5 and 6) suggest that metastasis to bone marrow may require very different cellular characteristics than those commonly studied in relation to lung metastasis. In addition, the ability to obtain bone marrow from animals as a suspension of largely single cells lends itself to the isolation of rare metastatic cells to allow examination of transient characteristics of the metastatic phenotype (7).

Animal models of metastasis to bone marrow and bone are needed to study this process. However, studies of metastasis in animal models have focused on pulmonary metastasis (8–10). The lung colony experimental metastasis model is widely used. In this model, tumor cells are injected in the mouse tail vein. Most of the cells die, but a few survive, grow, and form tumor colonies in the lung. The colonies are counted to quantify the experimental metastasis. The ability of the lung to kill the vast majority of injected tumor cells (11, 12) may preclude the use of i.v. injection to study metastasis to extrapulmonary organs. Bone metastasis models have involved direct injection of tumor cells into the medullary cavity of bones (13, 14) or into the rat abdominal aorta (15). In this paper, we describe our experience with an animal model in which murine B16 melanoma cells reproducibly colonize specific sites of the skeletal system of syngeneic animals. Tumor cells were injected into the left cardiac ventricle, thus bypassing the pulmonary vasculature. All bone metastasis occurred in bone adjacent to hematopoietic bone marrow.

MATERIALS AND METHODS

Animals. Female C57BL/6 mice (Charles River, Wilmington, MA) aged 8 to 16 wk were used for all experiments. All animals were maintained under the guidelines established by NIH and the University of Rochester.

Tissue Culture. The G3.26 cloned subline of B16 murine melanoma (8) was the generous gift of Christopher Stackpole. This subline was selected for its marked capacity to colonize the lung after i.v. injection. Cells were grown in 50% Dulbecco's modification of Eagle's medium-50% Ham's F12 medium containing 15% fetal bovine serum, 1.2 g of $NaHCO_3$/liter, 3.57 g of *N*-2-hydroxyethylpiperazine-*N'*-2-ethanesulfonic acid, 50 units/liter of penicillin, 50 μg/liter of streptomycin, and 2 mM glutamine in a humidified gas mixture of 5% CO_2 and 95% air at 37°C. Cells were harvested by brief exposure to a solution of 0.1% trypsin and 0.02% EDTA in calcium- and magnesium-free phosphate-buffered saline. All tissue culture materials were purchased from Gibco, Grand Island, NY. Growing cultures were passaged just prior to or upon reaching confluence, and they were replaced from frozen stocks every 4 to 6 wk.

Injection into Animals. All cultures used for injection were subconfluent and were fed the day prior to use. The harvested cell suspension was washed twice by centrifugation in medium with serum at room temperature and resuspended in medium without serum at 4°C immediately prior to injection. The number of cells to be injected was suspended in 0.1 ml. Cell viability was determined by trypan blue exclusion, and only cell suspensions with greater than 95% viability and without cell clumping were used. The cell suspension was agitated prior to each syringe being filled for each injection.

For i.v. injection of tumor cells, mice were warmed 10 in away from a 150-W light bulb for 20 to 30 min. Animals were then immobilized in a restraining device, and cells were injected into a lateral tail vein. Animals were sacrificed 3 wk after injection. For intracardiac injection into the left ventricle, animals were anesthetized by inhalation of methoxyflurane. The anterior chest wall was shaved and scrubbed for aseptic surgery by washing with iodine and 70% alcohol. The skin on the chest was opened with a 1-cm midline incision to expose the ribs. A 30-gauge needle on a tuberculin syringe was then inserted in the second intercostal space 2 mm to the left of the sternum and aimed centrally. The spontaneous, pulsatile entrance of bright red oxygenated blood into the transparent needle hub indicated proper positioning of the needle. A suspension of 10^2 to 10^5 tumor cells in 0.1 ml was injected over about 1 min. Animals were sacrificed by ether inhalation when they became ill or paralyzed. Viscera were removed, and viscera and carcass were fixed in 10% formalin for subsequent scoring of metastasis and histological examination.

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Scoring of Metastases. All organs including skin and muscles were examined carefully. Pigmented metastatic colonies were easily seen and counted on the surfaces of organs with the aid of a dissecting microscope. In some studies, interior organ metastasis was sought by sectioning. Scoring of skeletal metastasis is described in "Results" below. Histological examination confirmed that less than 1% of tumor colonies in all organs were amelanotic. Only melanotic colonies were counted.

Histological Examination. After removal of the pelt, viscera were removed, and the viscera and skeleton were fixed in 10% phosphate-buffered formalin (pH 7.2). Bones were decalcified for 5 to 15 h in a solution containing 0.7 g/liter of EDTA, 8 mg/liter of Na-K tartrate, 99.2 ml/liter 37% HCl, and 140 mg/liter of sodium tartrate. Bones were then washed for 48 h in running tap water, dissected, and trimmed into blocks. Standard techniques of paraffin embedding, sectioning, and staining were used.

Distribution of Bone Marrow in the Mouse. Relative distribution of bone marrow was established by simple observation of the reddish tinted hematopoietic areas observed when holding fixed bones up to a bright light. This bone marrow distribution was confirmed by histological examination.

RESULTS

Injection of Tumor Cells i.v. When 10^5 cells were injected i.v. in each of 20 mice, a mean of 30 lung tumor colonies per animal was found. No melanotic tumor colonies were found in the skeleton. Injection of 10^2 cells did not result in any melanotic tumor colonies in the lungs or extrapulmonary organs.

Intracardiac Injection of Tumor Cells. Injection of tumor cells in the left cardiac ventricle was tolerated remarkably well. About 10% of the mice died within 1 or 2 min following left intracardiac injection. All of the rest of the animals became sick 14 to 20 days after intracardiac injection, at which time they were sacrificed. An autopsy was performed on every mouse.

Distribution of Tumor Colonies in Organs Other Than the Skeletal System. Intracardiac injection of 10^5 melanoma cells resulted in the appearance of melanotic tumor colonies in most organs. When fewer tumor cells were injected, differences between organs in the incidence of tumor colonies became more apparent (Table I). Kidney, spleen, and brain were the organs least likely to have metastatic colonies. Lungs contained only a few tumor colonies. Tumor colonies were found in adrenal glands and lymph nodes to an intermediate degree. The skeleton and the ovaries were the organs most often affected and the only organs with tumor colonies when only 100 cells were injected. Metastatic tumors in the ovary were often massive (~1 cm) compared to the uniform size (~2 mm) of the tumor colonies usually found in other organs. However, tumor masses in the ovaries were usually cystic.

Table 1 *Organ distribution of melanotic tumor colonies after injection of B16 melanoma cells in the left cardiac ventricle*

Location of tumors[a]	No. of mice with tumor colonies/total mice with the following nos. of tumor cells injected					
	1×10^5	5×10^4	1×10^4	5×10^3	1×10^3	1×10^2
Bone/bone marrow	10/10	5/5	5/5	5/5	4/5	3/5
Lungs	10/10	2/5	1/5	0/5	1/5	0/5
Liver	6/10	1/5	0/5	0/5	0/5	0/5
Pancreas	4/10	1/5	0/5	0/5	0/5	0/5
Ovaries	10/10	4/5	4/5	5/5	1/5	2/5
Uterus	5/10	0/5	0/5	0/5	0/5	0/5
Spleen	2/10	0/5	0/5	1/5	0/5	0/5
Adrenals	10/10	5/5	4/5	5/5	3/5	0/5
Kidneys	1/10	0/5	0/5	0/5	0/5	0/5
Brain	3/10	1/5	1/5	0/5	1/5	0/5
Skeletal muscle	7/10	2/5	0/5	2/5	0/5	0/5
Skin	6/10	1/5	0/5	0/5	0/5	0/5
Others[b]	10/10	5/5	5/5	5/5	1/5	1/5

[a] Whole organs were analyzed for macroscopically visible melanotic nodules 14 to 20 days after left intracardiac injection of B16 melanoma cells.
[b] Mesentery, thymus, lymph nodes.

Tumor Colonies in the Skeletal System. Tumor colonies were found in the bone and bone marrow of all mice given injections in the left cardiac ventricle with 5×10^3 or more tumor cells. The observed sites of bony metastasis are displayed in Fig. 1*a*. For comparison, the location of bone marrow in animals of the same age and sex is shown in Fig. 1*b*. Melanotic tumor colonies were most commonly found in the axial skeleton, including cranium, mandible, maxilla (Fig. 2), vertebral bodies (Fig. 3), and pelvis. Metastases were also commonly found in the proximal large bones of the extremities. No metastases were ever seen in the most distal small bones such as carpals and tarsals (Table 2). No animals had involvement of all possible bone sites. Tumor-containing and tumor-free vertebral bodies were often found adjacent to each other. Also, tumor colonies were present in specific regions of each bone. In the long bones bilateral melanotic tumor colonies were situated at the proximal ends, except for the femur where tumor was found in the distal end (Fig. 4). Metastases usually were not seen in the middle of the shaft of the long bones. In flat bones, such as ischium, ilium, and scapula, bilateral melanotic tumor colonies were situated in the periphery. When the ribs were affected, the melanotic tumor was limited to the distal ends, stopping at the costochondral junction. Metastases to the spine were common events (Table 2) with the thoracic and lumbar regions being the most often affected followed by the sacral region.

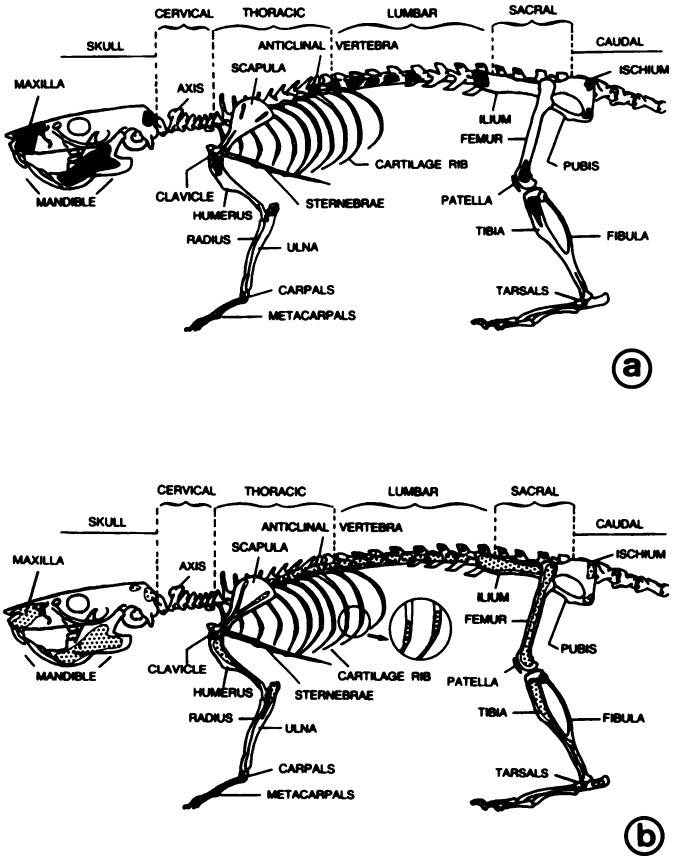

Fig. 1. *a*, location of tumor colonies in the skeletal system after injection of B16 melanoma cells into the left cardiac ventricle of female C57BL/6 mice. Mice were 8 wk old when given injections and 10 wk old when sacrificed. The *shaded areas* represent the regions where melanotic tumor cell colonies were most frequently seen. *b*, location of hematopoietic bone marrow in the skeletal system of a 10-wk-old female C57BL/6 mouse. Distribution was determined as described in "Materials and Methods." *Shaded areas* represent the major areas of hematopoiesis.

Fig. 2. Skull of mouse showing melanotic infiltration (*arrows*) into maxilla and mandible by B16 cells injected into the left cardiac ventricle.

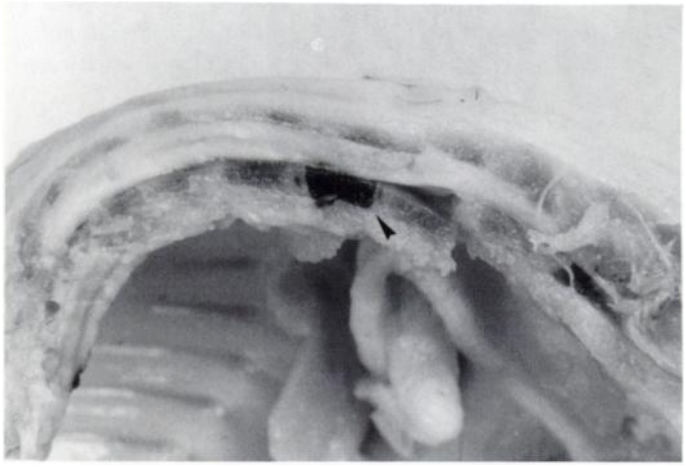

Fig. 3. Melanotic nodule of B16 cells growing in a lumbar vertebral body (*arrow*). This lesion produced spinal cord compression in the mouse (see text).

Injection of fewer than 5×10^4 tumor cells resulted in selective colonization of certain bones (Table 2). The thoracolumbar spine, femur, tibia, maxilla, mandible, and pelvis were the most commonly affected. The pattern of metastasis within individual bones did not change with the number of tumor cells injected.

Histological examination of bone metastasis demonstrated masses of melanin-containing cells. In every case, the melanoma cells displaced or replaced normal hematopoietic bone marrow (Fig. 5*a*). Some tumor cells passed through the invaded bone to the surrounding muscular or neural tissue (Fig. 5*b*).

Quantitation of Bones with Tumor Colonies. The number of bones with melanotic tumor colonies was counted in each animal. The number of bony sites involved per animal ranged from 0 to 43 of 56 bony sites examined. The more tumor cells injected, the more bony sites were found with melanotic tumor colonies. Injection of 100 tumor cells resulted in occasional tumor colonies in bones of most mice. Injection of 5000 tumor cells resulted in tumor colonies in bones of all mice (Table 1). A total of 23 sites in the axial skeleton, proximal tibia, and distal femur was most commonly colonized by tumor (Table 2). These were examined in each mouse. The results (Fig. 6) showed an increase in the number of bones colonized by tumor as the number of injected cells increased. This relationship was complex and biphasic. Between 100 and 10,000 cells injected per animal, there was a 3-fold increase in tumor colonies for

Table 2 *Skeletal distribution of melanotic tumor colonies after left intracardiac injection of B16 melanoma cells*

	No. of mice with tumor colonies/total mice with the following nos. of cells injected					
	1×10^5	5×10^4	1×10^4	5×10^3	1×10^3	1×10^2
Spine (region)						
Cervical	0/10	0/5	0/5	0/5	0/5	0/5
Thoracic	10/10	4/5	4/5	2/5	1/5	0/5
Lumbar	9/10	4/5	4/5	3/5	1/5	1/5
Sacral	5/10	2/5	2/5	1/5	0/5	1/5
Skull						
Cranium	7/10	4/5	4/5	0/5	1/5	0/5
Maxilla	10/10	5/5	5/5	5/5	4/5	0/5
Mandible	10/10	5/5	5/5	4/5	3/5	1/5
Thorax						
Ribs	10/10	3/5	0/5	0/5	0/5	0/5
Sternum	4/10	3/5	0/5	0/5	0/5	0/5
Scapula	9/10	5/5	4/5	0/5	1/5	0/5
Pelvis	10/10	4/5	5/5	2/5	2/5	1/5
Limb						
Foreleg						
Humerus	10/10	4/5	1/5	0/5	2/5	0/5
Ulna	3/10	3/5	0/5	0/5	0/5	0/5
Radius	0/10	0/5	0/5	0/5	0/5	0/5
Carpals	0/10	0/5	0/5	0/5	0/5	0/5
Hind leg						
Femur	10/10	5/5	5/5	4/5	2/5	1/5[a]
Tibia	10/10	5/5	5/5	4/5	1/5	1/5[a]
Fibula	4/10	2/5	2/5	0/5	0/5	0/5
Tarsals	0/10	0/5	0/5	0/5	0/5	0/5

[a] Unilateral.

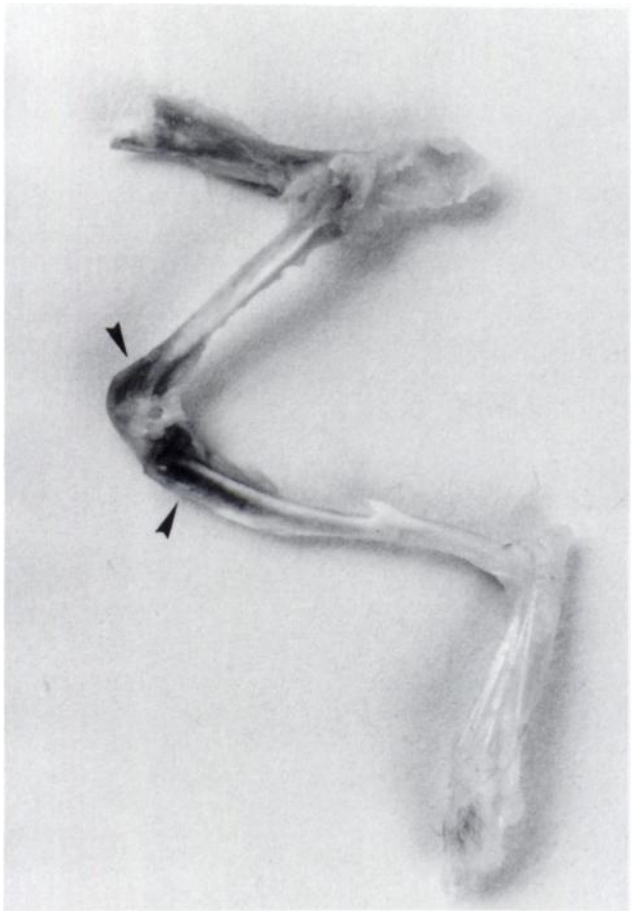

Fig. 4. Mouse hind quarter showing melanotic tumor colonization of distal femur and proximal tibia (*arrows*).

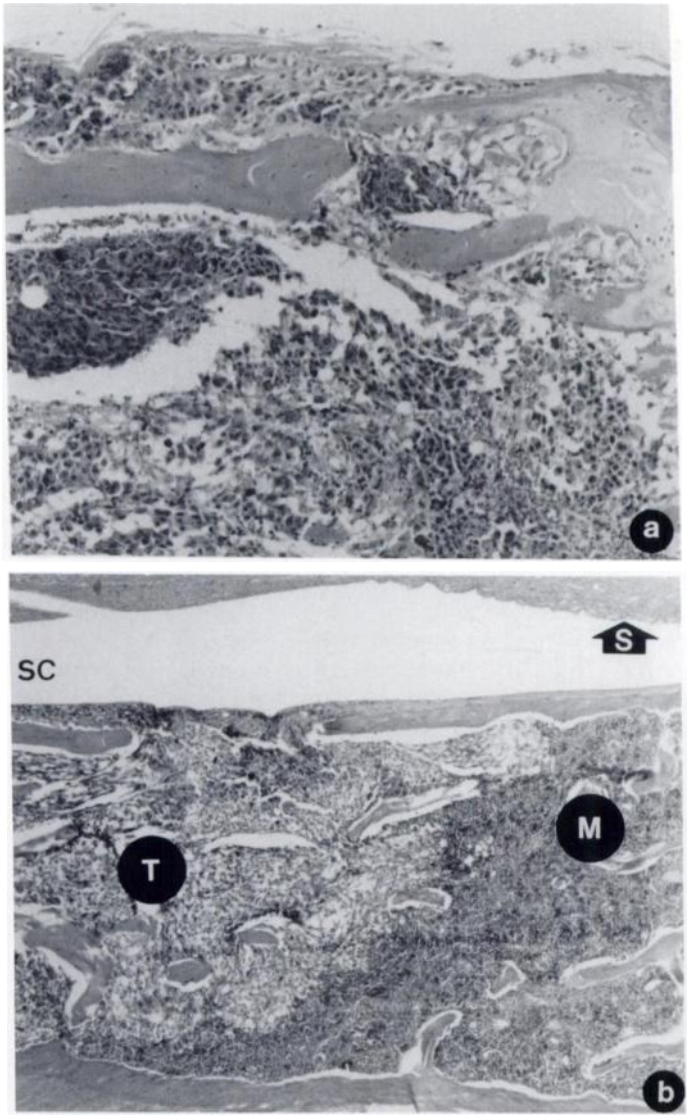

Fig. 5. *a*, tumor colonization of a distal femur. Note invasion of the tumor cells through the cortex. *b*, tumor colonization of a vertebral body. Melanotic tumor cells (*T*) have replaced part of the normal hematopoietic marrow (*M*) and invaded the spinal canal (*SC*). *S* marks the spinal cord.

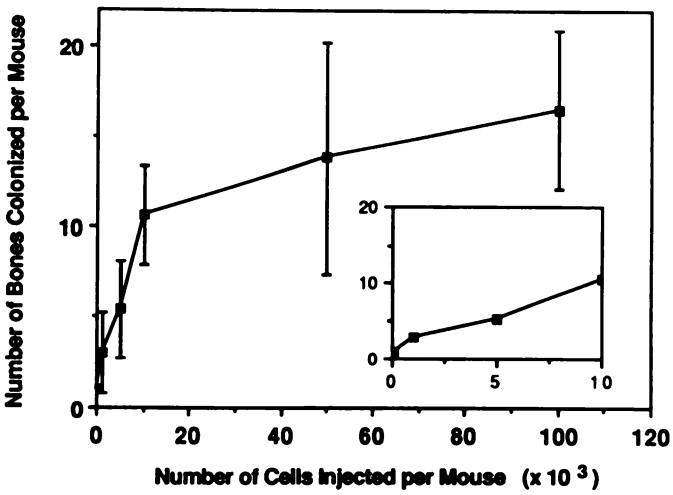

Fig. 6. Effect of number of injected cells on number of bone colonies. Fifty-six bony sites were examined for macroscopically visible melanotic colonies 14 to 20 days after tumor cell injection. *Points*, mean number of bony tumor colonies in 5 mice. *Inset*, magnification of the curve between 10^2 and 10^4 cells injected per mouse.

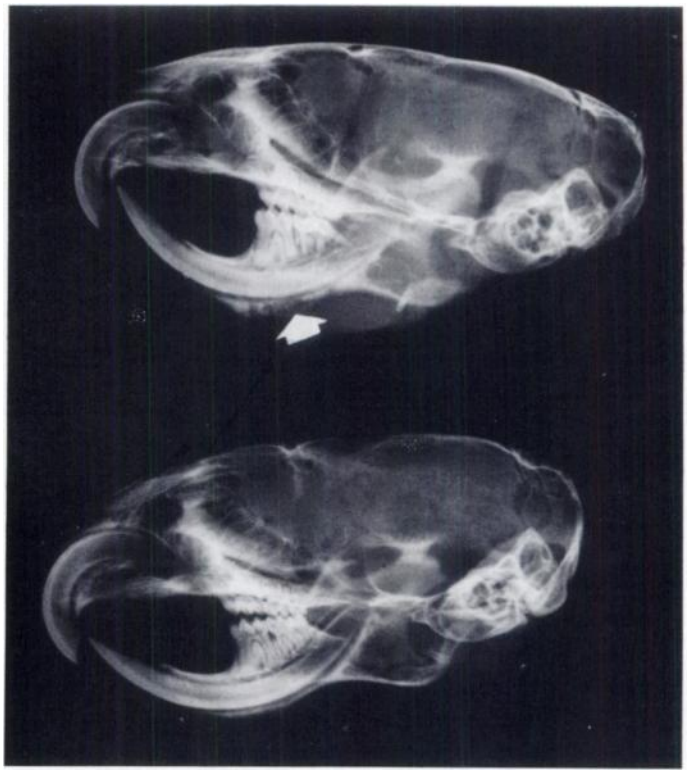

Fig. 7. Radiographic appearance of bone metastasis. The *upper skull* shows the destruction of a mandible colonized by B16 melanoma (*arrow*). A normal mandible is shown in the *lower skull* for comparison.

each 10-fold increase in the number of cells injected. A further 10-fold increase from 10^4 to 10^5 cells injected resulted in only a few more tumor colonies in these bones (Fig. 6).

Clinical Findings. After receiving injections of 5×10^3 or more tumor cells, almost all animals developed paralysis of the hind legs. These animals also had an enlarged bladder due to urinary retention. Both of these findings resulted from compression of the spinal cord by a melanotic mass of tumor cells growing from thoracic or lumbar vertebral bodies (Fig. 3). A few animals became moribund and were found to have massive blood loss into the peritoneal cavity associated with large tumor colonies in the ovaries.

Diagnosis of melanotic bone metastasis could be presumed in most of the living, nonparalyzed animals by the observation of pigmentation in the gums. The melanotic infiltration around the base of the teeth was contiguous with mandibular bone colonization (Fig. 2). In animals with advanced metastatic disease, the radiographic appearance of bone destruction was most clearly demonstrable in the mandible (Fig. 7).

DISCUSSION

We have developed a murine model of metastasis to bone marrow and bone. We used melanoma because of its propensity to metastasize to a wide variety of organs, producing pigmented colonies which can be readily distinguished from the organ parenchyma. We chose a B16 melanoma clone that had been selected for its ability to cause many pulmonary colonies after i.v. injection. Injection into the left cardiac ventricle resulted in tumor colonies in most organs of the mouse. Injection of progressively fewer cells resulted in selective colonization of the ovary and bone marrow. The skeletal system appears to be

more able than other organs to support growth of the injected B16 G3.26 tumor cells. Indeed, bone metastasis were remarkably common and widespread.

Injection of 10^5 tumor cells i.v. resulted in lung tumor colonies only, whereas intraarterial injection 10^5 cells resulted in colonies in essentially all organs of the body, but very few colonies in the lung. Because arterial injection of 10^2 cells caused bone colonization and i.v. injection of 10^5 cells caused none, less than 10^2 (0.1%) of the i.v. injected cells must have passed viably through the lung and into the arterial circulation. Thus, the injected tumor cells appear to arrest in the first capillary bed in which they arrive. This hypothesis is supported by the findings that the vast majority of i.v. injected tumor cells rapidly arrest in the lung (11), and that arterially injected embryonal carcinoma cells appear to arrest immediately in capillary beds (16). It seems likely that B16 cells must have reached all tissues when 10^5 cells were injected into the left cardiac ventricle. The low incidence of tumor colonies in the kidney, spleen, and brain might be due to failure of the B16 cells to survive vascular arrest in those organs, invade into organ parenchyma in order to escape intravascular host defenses, or to proliferate in the environments of those organs. In spontaneous metastasis to extrapulmonary organs, some cells must pass through the lung capillary bed into the arterial supply and lodge in other organs. A major role of the lung capillaries in metastasis may be to prevent metastasis by trapping and mechanically destroying almost all the tumor cells that enter the venous circulation (12).

Others have noted that the route of injection of tumor cells into the blood circulation has a dramatic effect on tumor colonization (17–19). For example, intraportal injection of Walker carcinoma cells most frequently results in liver metastasis only, whereas i.v. injection leads to extensive lung metastasis but not to involvement of other organs, including the liver (20).

Bones with melanotic tumor colonies were those rich in hematopoietic bone marrow. There were no colonies of B16 melanoma in bones that did not have bone marrow. The presence of bone marrow also appears to be a prerequisite for the establishment of human bone metastasis (4, 14). Bone marrow appeared to be the organ that best supported tumor colonization, although the ovary also was a common site of experimental metastasis. Intracardiac injection of 10^2 cells caused bone marrow colonization, but the same number of cells injected i.v. did not colonize the lung. Three essential steps in the process of metastasis—arrest, extravasation, and proliferation of the tumor cells—appear to be relevant to this model of bone marrow metastasis. Bone marrow vasculature is unique in many aspects. All three major constituents of its vascular tree—arteries, sinusoids, and veins—exhibit morphological modifications that are presumably structural adaptations to the functional needs of the bone marrow (21). The arterioles become sinusoids without an intervening capillary (22), which might decrease intravascular mechanical destruction of tumor cells. The alternate flow and stagnation of blood in the sinusoids (23) might facilitate tumor cell arrest. The thin sinusoidal walls have fenestrations and areas free of basement membrane (24, 25), which may more readily permit extravasation of tumor cells. The last stage of metastasis, *i.e.*, the proliferation of the tumor cell at the site at which it lodges, may be dependent upon the capacity of the cells to recognize local, tissue-specific growth signals. With regard to bone marrow, cultured bone marrow stromal cells stimulate the *in vitro* growth of cultured carcinoma cells (6). Also, leukemia cells may have "homing" receptors that function not only to promote adherence to bone marrow stroma, but also to support leukemia cell survival and growth in the bone marrow environment (5). It seems unlikely that "homing" receptors are relevant to this model of solid tumor metastasis because the injected cells appear to lodge in the first capillary bed that they meet. However, the ability of bone marrow to support tumor cell colonization deserves further investigation.

The pattern of skeletal distribution of tumor colonies closely mimics the pattern of metastasis to bone in humans (4). The pattern of colonization within each bone was highly predictable. Colonization occurred only in the distal femur and only in the proximal areas of the humerus, tibia, ulna, and fibula. Usually, none of these bones developed metastatic tumor colonies in the midshaft region. Bone marrow was plentiful in midshaft regions, so this would not explain differences in metastatic site. In humans, the area of a long bone most commonly involved by metastatic processes is the metaphysis (26). The metaphysis receives much more blood flow than the other regions of the bone (25), and the difference in the number of tumor cells delivered to this region might cause the observed difference in distribution of tumor colonies. It is also possible that the different regions of bone marrow have varying ability to support tumor colonization. In the lung, B16 colonies are found almost exclusively on the pleural surface. Such extreme intraorgan variation in tumor colonization suggests that there may be major variations within an organ that may determine whether or not tumor colonization may occur.

The relationship between the number of bony sites colonized and the number of cells injected was consistent when 10^4 or fewer cells were injected. When the number of injected cells was increased beyond 10^4, proportionately fewer additional bone tumor colonies were seen. This is in contrast to the lung colony experimental metastasis model, in which the number of lung metastases is proportional to the number of tumor cells injected (20, 27). The lack of proportional increase in the number of bone tumor colonies may result from more than one colonizing cell participating in the formation of some colonies. Alternatively, each additional site colonized may require additional or different colonizing cell characteristics that are found only in progressively rarer cells. The complex quantitative relationships found do not appear to fit a simple model, and further investigations are planned.

We have also observed bone metastasis in animals given injections in the left cardiac ventricle with cells of murine breast and lung carcinoma lines and with a different subclone of B16 melanoma that is poorly metastatic to the lung (data not shown). It may be that bone colonization occurs with many clones of B16 melanoma and other metastatic tumor cell lines, but because most experimental models study cancer metastasis after i.v. injection and because bones are not examined, bone metastases have not been reported.

The utility of this model for future studies is clear. The pattern of colonization of the bone is highly reproducible. Melanotic metastasis to the bone is easy to quantitate, requiring little more effort than counting of tumor colonies in the lung. There are two clinical endpoints which may facilitate studies, melanotic pigmentation of gums and development of paralysis due to spinal cord compression. The ability to quantify experimental metastasis to the bone marrow and bone will allow this model to be used to study the effects of a variety of factors on the ability of injected tumor cells to colonize bone. The ease of preparation of bone marrow as a single cell suspension may

simplify the isolation of colonizing cells at various stages in their proliferation.

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