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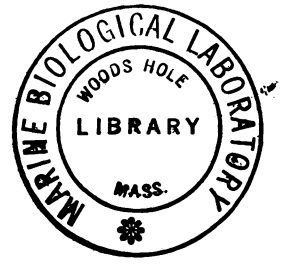

Do Metastases Metastasize?

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IT IS well known that metastases form from tumor cells released from primary tumors. However, the role of established metastatic foci in the further dissemination of cancer is unclear. Experimentally it has been shown that after amputation of a primary tumor established pulmonary metastases shed viable cells as demonstrated by injecting left heart blood containing tumor cells into normal syngeneic recipients.[7] Although these cells retain their growth potential in normal syngeneic hosts, the critical question pertains to the true metastatic potential of such cells in the autogenous host. It seemed possible that tumor-specific immunity elicited by growth of the primary tumor,[9] and perhaps facilitated by its amputation, might render these secondarily circulating tumor cells biologically impotent.

The phenomenon of preferential metastases, i. e., a specific trophism of certain tumors for a given tissue,[8] although poorly understood on a cellular level, seemed a useful tool for investigating the biologic potential of cells shed from pulmonary metastases. By timing the placement of heterotopic isologous lung transplants before or after amputation of a "lungphilic" primary tumor, data regarding the biologic potential of cells in the autogenous animal was obtained.

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Materials and Methods

The fifth generation of a 3-methylcholanthrene (MCA[5]) sarcoma induced in syngeneic female C57 mice was used. This particular MCA tumor metastasizes early and exclusively to lungs. Pronase enzymatic digestion of minced solid tumors produced single-cell suspensions. Cell viability was assessed by 0.25% trypan blue exclusion. Amputations of tumors were performed under pentobarbital anesthesia using the clamp and cautery method with autoclip skin closure. An occasional animal, developing local recurrence, was excluded from study.

Newborn transplants of kidney, lung, and spleen were placed subcutaneously by trocar as has been described in detail elsewhere.[15] Four to 6 weeks after transplantation, 76 per cent lung, 56 per cent spleen,

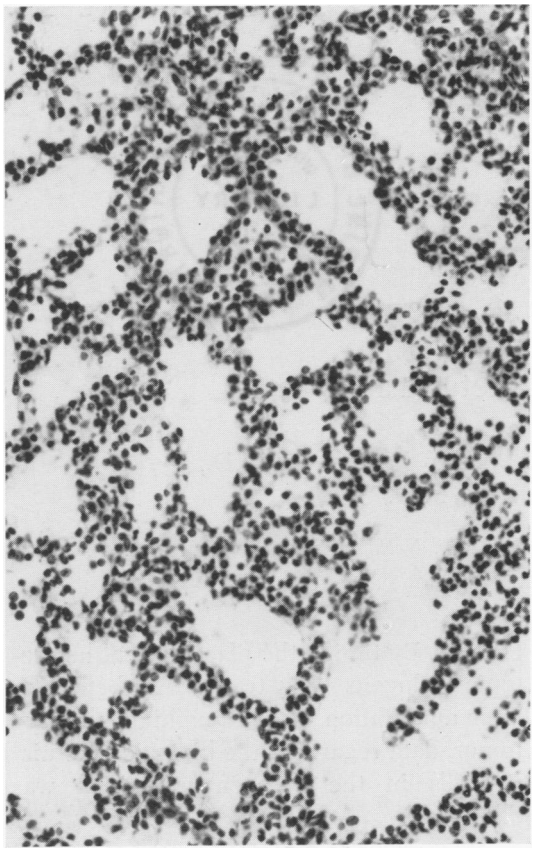

FIG. 1. Histology of newborn C57 mouse lung.

and 36 per cent of kidney transplants appeared viable after careful identification and removal using the dissecting microscope. Cytoarchitecture is altered by heterotopic transplantation (Figs. 1 and 2) but remains identifiable as the transplanted tissue.

Tumor specific immunity was induced by temporary tumor growth of the sixth transplant generation followed by amputation. Immunized and control animals were then challenged with graded doses of viable tumor cells subcutaneously. This tumor is highly immunogenic with significant inhibition ($p < 0.05$) of increasing challenge doses through 1.3×10^6 cells which grow in 100% of controls.[15]

Experiment I, Figure 3. Syngeneic mice were inoculated with 10^6 tumor cells into the right distal thigh. 48 hours later, mice received transplants of lung into the left leg, spleen into the left back, and kidney into the right back. A second group, was traumatized with the trocar or received 0.01 cc. of 10% formalin intramuscularly. Eighteen days after inoculation primary tumors reached 1.5–2.0 cm. diameter and were amputated to prolong survival. Pulmonary metastases are present in 70–80% of animals at this time in this tumor system. Twenty days after amputation mice were sacrificed and transplants were identified with the dissecting microscope and histologic confirmation obtained. Lungs were also examined for metastases with the dissecting microscope although nearly all tumors were easily visualized grossly.

Experiment II, Figure 4. Mice were inoculated with 10^6 tumor cells and 20 days later the 1.5–2.0 cm. tumors were amputated. 48 hours *after amputation* mice received A) two lung transplants into the left leg and right back, B) lung to left leg, spleen to left back, and kidney to right back, C) formalin into the left leg, D) no treatment. *Twenty-eight* days after transplantation animals were sacrificed and autopsies were performed. The untreated group was anesthetized and in animals *with* pulmonary metastases left heart blood was aspirated and injected intramuscularly into eight syngeneic recipients.

Results

Experiment I. Pulmonary metastases developed in 16/18 animals surviving the entire period without local recurrence. No other visceral metastases were present. Twenty days after tumor inoculation (at the time of amputation) six small 1–2 mm. nodules were palpable at the lung transplant site in the left leg. In mice with pulmonary metastases there were 7, 9, and 6 viable transplants of lungs, spleen, and kidney identified histologically. The six with nodules at lung transplant sites grew progressively, and, at sacrifice all proved to be fibrosarcomas with histology identical to that of the 1° lesion. Remnants of lung

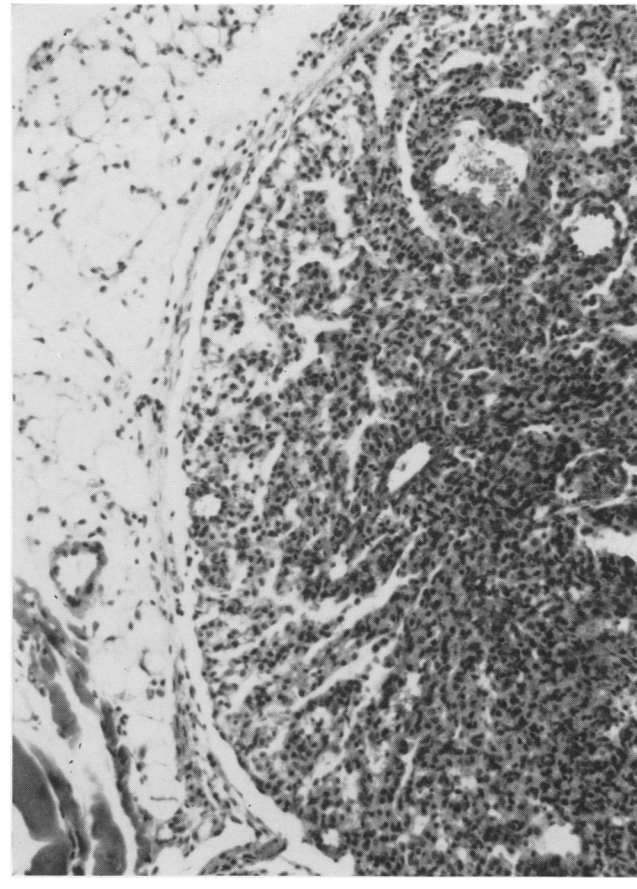

FIG. 2. Histology of a subcutaneous lung transplant after 6 weeks *in vivo*.

tissue could not be identified in these tumors. Spleen, kidney, and inflammatory or trocar control sites were histologically free of tumor.

Experiment II. In combined groups A and B 28/35 mice completing the study without local recurrence had pulmonary metastases. No other metastases were present. In these mice with pulmonary metastases there were 23, 8, and 6 identified transplants of lung, spleen, and kidney, respectively, and free of tumor on serial histologic section. Inflammatory sites were also negative. 8/8 or 100% of the bioassay recipients of group D developed tumor.

Discussion

Resection of solitary pulmonary metastases when a primary tumor is controlled is accepted as an effective curative surgical procedure with reported 5-year survivals of 30% [18] and 26% [19] for a combination of histologic types. One of the critical problems facing the surgeon who is evaluating a patient with one or several pulmonary metastases is the inability to detect more extensive microscopic metastases in the lungs or elsewhere. Lung tomograms can only detect lesions with a resolution to one cm. with less reliable tests for liver metastases. For these reasons a diagnostic observation period may be decided upon which allows for the growth of such subclinical metastases.

The question then, in terms of a solitary pulmonary metastasis, becomes succinctly phrased: "Do metastases metastasize?" If the question could be answered definitely affirmative, the 2 to 3-month diagnostic observation period might be too long since cells disseminated from the metastases would have true metastatic potential in

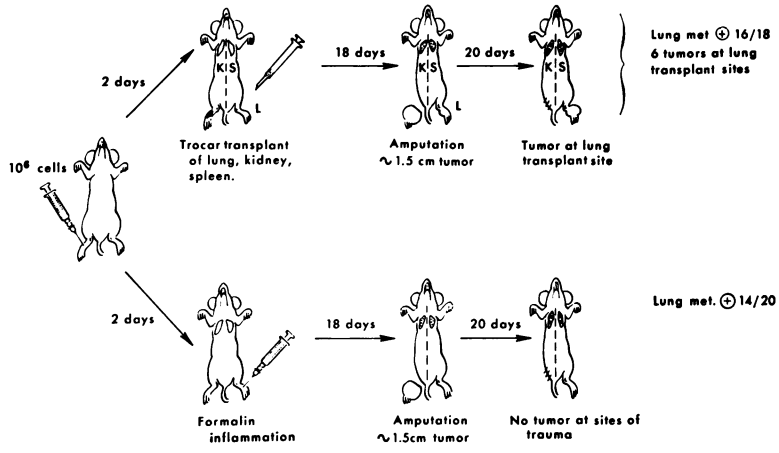

FIG. 3. Design of experiment I with experimental results at time of sacrifice. Preferential metastasis to pulmonary tissue was demonstrated.

the autochthnous host. If negatively answered, this interval certainly does allow for military deposits of microscopic disease at least in the lung to become grossly manifest and avoids unnecessary thoracotomy in some patients.

Ketcham *et al.*[7] reported the bioassay of viable tumor cells in three transplantable tumor systems shed from pulmonary metastases. Animals had been allowed to grow large primary tumors which metastasized regularly and selectively to the lungs. Tumors were then amputated, and, 4 to 6 weeks after amputation left heart blood was aspirated from animals with pulmonary metastases and injected into inbred-syngeneic mice. Recipient mice developed tumors in all three systems demonstrating that viable tumor cells are shed from pulmonary metastases.

An accumulating body of evidence, however, suggests that tumor-specific immunity elicited by growth of the tumor can participate in the control of metastasis formation from circulating tumor cells.[2, 5, 6, 15] Antilymphocyte serum [4, 6] cortisone [1, 3, 16] and other immunosuppressants [16] facilitate metastases formation to organs not part of the general metastatic pattern of a tumor, or, cause metastases from tumors which ordinarily do not disseminate. Martinez *et al.*[10] have shown that animals with pulmonary metastases were resistant to second subcutaneous inocula of tumor *after* the primary had been amputated, also suggesting that immune resistance was present. We [17] have shown that in at least one experimental system, that metastases were immunogenically different from the primary tumor from which they originated. Immunity to circulating tumor cells could hypothetically provide for natural selection of immunologically altered cells for metastasis formation. Likewise, Alexander [2] demonstrated that rats with growing benzpyrene sarcomas can reject intravenous infusions of autochthnous tumor cells.

These experiments were designed to investigate the metastatic potential of cells shed from pulmonary metastases using a known immunogenic tumor system. By exposing heterotopically transplanted lung tissue, the site of preferential metastases in this system, to viable tumor cells shed either from the primary tumor or from the pulmonary metastases, data pertaining to this question was obtained. Only animals with demonstrated pulmonary metastases were included in the results of Experiment I for only those animals with pulmonary metastases developed lung transplant metastases. In Experiment II the pulmonary metastases were the only possible source of tumor cells, since the primary had been amputated. We assumed that the six animals developing tumors at the lung transplant site in Experiment I had viable lung transplants before being destroyed by tu-

mor. This seems a reasonable assumption since no other transplant sites or trocar or formalin traumatic foci became involved. Therefore 6/13 lung transplants developed secondary metastases. In Experiment II transplants were exposed to tumor cells disseminated from pulmonary metastases, and none of 23 viable lung transplants developed metastases. Also of note is that in experiment I all six tumor nodules were palpable by 20 days and no additional tumors developed. In Experiment II, animals were followed for 28 days after amputation and even on serial section of transplants no tumor could be seen. Granted the assumption made before statistical comparison, these differences are significant by Chi [2] test to $p < 0.01$ level.

These data show that in this immunogenic murine tumor-system viable cells released from pulmonary metastases would not form secondary metastases to heterotopically placed lung transplants, a site of demonstrated preferential metastasis. In addition, all lung metastases in the animals four weeks after amputation were of the same size, as was also observed by Ketcham [7] suggesting that even orthotopic lung was not re-seeded by tumor cells disseminated from these metastases. The bioassay of left heart blood definitely demonstrated that large numbers of cells were present in circulating blood, a finding confirming observations of other investigators [7, 13] in animals with metastases.

Since this tumor has been demonstrated highly and specifically immunogenic,[15] an observation consistent with those of Prehn and Main,[11] Sjernwald,[14] Old and Boyse [12] and others [9] made regarding methylcholanthrene-induced murine tumors, it is reasonable to hypothesize that tumor-specific immunity developing after exposure to the growing primary, and perhaps facilitated by its removal,[14] was effective in controlling growth of these cells even in sites of preferential metastasis. Alternatively, but seemingly less likely, the cells selected for first order metastasis may be a subpopulation of the primary tumor population which are not capable of re-metastasizing autogenously. The fact that left heart blood from animals with pulmonary metastases infused intravenously produces lung tumors in syngeneic recipients [7] mitigates against this possibility. In another system (benzpyrene-induced sarcoma—inbred Fisher rat), it has been demonstrated that spontaneous metastases from tumors derived from metastatic cell lines have the same propen-

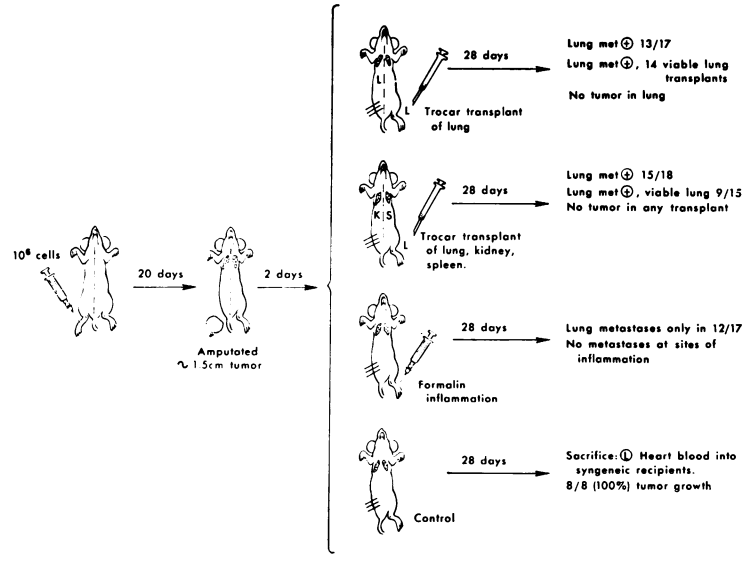

FIG. 4. Design of experiment II with results in each group at the time of sacrifice. When transplants were placed after amputation of the primary, no metastases formed at lung transplants.

sity for spontaneous metastasis as did the primary.[17]

Direct extrapolation of data from these few animal tumor-systems to the clinical situation is not possible. However, experimental data can provide useful information giving direction to clinical research. These data indicate that in an isologous highly immunogenic experimental tumor system, pulmonary metastases do not remetastasize to sites of preferential metastasis even though they shed significant numbers of viable tumor cells. Development of tumor specific immunity after exposure to the primary, perhaps facilitated by removal of the primary, would seem a plausible control mechanism. In this system, the "diagnostic observation period" would not have prohibitively increased the risk of further dissemination and, by allowing other subclinical metastatic disease to become grossly manifest unnecessary surgery would be avoided.

Summary

A study was undertaken to investigate the biologic potential of tumor cells shed from pulmonary metastases in the autogenous host. First, preferential metastasis of the 5th generation of a methyl-cholanthrene-induced tumor of syngenic C57BL mice to lung tissue was demonstrated by subcutaneous transplantation of organ fragments. In a second experiment lung was transplanted into animals with pulmonary metastases. No secondary metastases occurred. Since this tumor is highly immunogenic, it was hypothesized that tumor specific immunity perhaps facilitated by amputation of the primary may inhibit the growth of tumor-cells shed from pulmonary metastases even in sites of preferential metastases. In this immunogenic murine tumor system, metastases do not metastasize.

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