ABSTRACT

The normal incidence of metastasis was determined in 207 C3H/He and 42 C3H/He mice with spontaneous mammary tumors. The effects of early versus delayed surgical removal of the tumors on the incidence of metastasis were studied in the C3H/He mice. The presence of metastases was determined by histological examination, primarily of the lungs. The incidence of metastasis was proportional to the size the primary tumors were allowed to reach before surgery, with the highest incidence in mice not surgically cured.

INTRODUCTION

There is much information from laboratory investigations using tumors selected for heterotopic growth and metastasizing characteristics on the mechanics of the extravasation, the dissemination, the arrest, and the extravasation of cells from implanted tumors or cells injected in suspension (1). There is, however, information only on the incidence of naturally occurring metastases derived from the study of autochthonous tumors (2–5).

Because the mouse mammary tumor model is one of the most commonly used models in cancer research, it seemed important to try to establish, for the first time, some basic facts about spontaneous metastasis from autochthonous spontaneous tumors in situ. Therefore, the purpose of this investigation was to determine the incidence of metastasis when the mice were surgically cured of spontaneous tumors of different sizes and also when mice carried their first spontaneous tumor to a near-terminal stage. The tissue of tumor origin (mammary epithelium or mammary stroma) and the immunogenicity of randomly selected metastasizing tumors were also determined. With this survey, it was hoped to learn about the incidence of unassisted metastasis from autochthonous tumors and to learn about the influences on metastasis of tumor size, tumor characteristics, host age, and the length of survival from the appearance of the first tumor.

MATERIALS AND METHODS

Mice. The tumor-producing mice were breeding or retired pedigreed females of 2 sublines of the C3H strain, the exogenous (milk transmitted) mouse mammary tumor virus-infected C3H/He and the aforementioned virus-free C3H/He. To ensure genetic similarity between the 2 sublines, the C3H/He line had been rederived from pedigreed C3H/He mice in 1973, 1976, and 1979 by foster-nursing on C3H/He mice.

The mice used in tumor immunogenicity studies were 8–10-week-old line-bred female C3H/He and C3Hf/He mice.

Surgery. Primary mammary tumors and implanted tumors were excised with the use of an ophthalmic platinum-tipped cautery and with wide excision margins to prevent recurrences. The primary C3H/He tumor hosts were selected to tumor size categories of 5, 9, and 12 mm in diameter, by random choice. Tumors were removed from mice under the short-acting methoxyflurane inhalation anesthetic Penthrane (Abbott Laboratories, N. Chicago, IL). The skin incisions were closed with wound clips. Because C3H/He mice have a high incidence of tumors, the mice in the surgery categories were subjected to repeated surgery (see Table 2). Each primary tumor host, in surgery and in nonsurgery categories, was killed by CO2 asphyxiation when the mouse appeared moribund.

Immunogenicity Tests. Tissue from pulmonary metastases and also from the primary tumor when the origin of the metastases was certain was implanted into the right No. 4 mammary glands of 10 mice using an 18-gauge biopsy needle. After a period of growth (usually to 10 mm), the immunizing tumor was removed under Penthrane anesthesia. Directly after tumor removal, the 10 cured mice and 10 normal mice received implants of tissue from the removed tumors into the left No. 4 mammary gland. Tumor growth was measured twice weekly with calipers. Each test was terminated (CO2 asphyxiation) when any of the mice reached an early stage of cachexia. The tests were repeated 1–3 times, using tumor tissue from one of the mice in the normal control groups.

Histology. The lungs of all of the primary tumor hosts, and other organs when suspected of containing metastases, were examined histologically. The specimens were formalin fixed and paraffin embedded. Stepwise, 3-μm serial sections were taken of each of the 5 lobes of the lungs. Organs which were only rarely the site of metastasis (ovaries, adrenals, liver, draining lymph nodes) were in most cases only inspected by gross examination at autopsy. The histological stain was hematoxylin-eosin. The quantity of metastases found by gross examination at autopsy and in histological sections was graded on a scale from 1 to 5 shown in Table 1.

Keratin and Vimentin Assays. The cellular origins of metastasizing and nonmetastasizing mammary tumors were determined by keratin content (adenocarcinomas of epithelial origin) and vimentin content (sarcomata of histioyte or fibrocyte origin) of the cells in frozen sections of primary tumors and grossly visible pulmonary metastases. The assay procedures were described in a recent publication (6)

Statistical Analysis. Differences in tumor incidences and metastasis incidences were evaluated with the χ2 test. Differences in mean tumor size, age at first tumor, and host survival were evaluated with Student's t test. Differences in the quantities of metastases found were evaluated with the Mann-Whitney test. Differences in the extravasating characteristics of tumors were evaluated with Fisher's exact test.

RESULTS

Metastasis. The results presented in Table 2 show that in C3H/He mice, the incidence of metastasis to the lungs was related to the size that the tumors were allowed to reach. There

Table 1 Quantitative grading of pulmonary metastases

<table>
<thead>
<tr>
<th>Grade</th>
<th>Description</th>
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<tbody>
<tr>
<td>1</td>
<td>1–3 small metastases (&lt;0.1 mm in diameter)</td>
</tr>
<tr>
<td>2</td>
<td>4–10 small or 1 medium (0.1–0.2-mm) metastases</td>
</tr>
<tr>
<td>3</td>
<td>&gt;10 small, or 2–5 medium, or 1 large (0.3–1.0-mm) metastases</td>
</tr>
<tr>
<td>4</td>
<td>&gt;5 medium, or &gt;1 large metastases</td>
</tr>
<tr>
<td>5</td>
<td>metastases visible grossly or with a dissecting microscope, and histologically confirmed</td>
</tr>
</tbody>
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was a significant difference in incidence between the 5- and 9-mm groups compared to the 11- and 28-mm groups. There was a general tendency for metastasizing tumors to develop earlier than nonmetastasizing tumors, but this distinction was significant (P < 0.05) only in the group of mice (Group 4) that carried their tumors without surgery. The metastasizing tumors in Group 4 were significantly slower growing (longer survival with similar mean tumor size at death) than the nonmetastasizing tumors (P < 0.001). There were no significant differences between the quantitative values of metastases between Groups 1 to 4.

Because of the differences in viral etiology, tumor incidence, tumor morphology, and age at tumor development between C3H/He and C3Hf/He mice (6), statistical comparisons were not made between the 2 sublines. It is noteworthy, however, that in the old C3Hf/He mice in Group 5, mammary tumors had a relatively low incidence of metastasis (24%) compared to the C3H/He mice in Group 4 (67%) and were relatively slow growing (long survival of tumor hosts).

Immunogenicity. The results presented in Table 3 show that of 16 metastasizing tumors that had been repeatedly tested for immunogenicity in syngeneic mice by intramammary immunization and challenge, 9 produced a significant level of protection against reimplantation of the same tumor. The primary tumors 41, 49, and 85 had the same immunogenicity as their metastases. This observation on the immunogenicity of spontaneously metastasizing tumors agrees with the results of earlier studies (7, 8). Those studies showed that the pulmonary transplantability (via tail vein injection) of syngeneic C3H/He mammary tumors was also not related to tumor immunogenicity.

Tumor Morphology and Pulmonary Growth. Of the 207 C3H/He tumor hosts studied, 91 were found to have metastases in the lungs (Table 2). All of the metastases found were type A (mainly acinar structure) or type B (varied structure, mainly nonacinar) (9) adenocarcinomas of epithelial origin (keratin positive, vimentin negative). None of the 91 metastasizing adenocarcinomas, including 7 that caused perivascular cuffing by monocytes and neutrophils at the sites of intravascular growth, showed any tendency to actively extravasate from pulmonary blood vessels but grew progressively within vessels (Figs. 1 and 2). Extravasation occurred only at points where blood vessels may have been ruptured by tumor expansion (Fig. 2).

Of the 42 C3Hf/He mice studied, 10 were found to have metastases in the lungs (Table 2). Four of the 10 tumors were type B adenocarcinomas and grew exclusively in the pulmonary vessels without active extravasation. The remaining 6 tumors were mammary sarcomas of histiocytic or fibrocyte origin (vimentin positive, keratin negative). In contrast to the adenocarcinomas, the pulmonary metastases from the sarcomas were always seen growing outside blood vessels (Fig. 3) and probably had the ability and tendency to actively extravasate soon after intravascular arrest.

**DISCUSSION**

The results presented in Table 2 show the following. (a) The incidence of lung metastasis in C3H/He Groups 1–4 was related to the size the tumors were allowed to reach. This observation differs from the conclusion reached by Price et al. (10) in their study of the metastatic potential of C3H/A mammary tumors. There was a significant difference in metastatic incidence (P < 0.01) only between Groups 1 and 2 versus Groups 3 and 4, suggesting that a critical tumor mass favoring metastasis was
reached somewhere between 9 and 12 mm. (b) The tumors that developed earlier had the greater tendency to metastasize, but only in Group 4 was the earlier development of metastasizing primary tumors statistically significant ($P < 0.05$). The significantly longer survival of the mice in Group 4A compared to Group 4B ($P < 0.001$) may have been a contributing factor in the histological detectability of some micrometastases. (c) The long survival and high tumor number and the very low incidence of metastases for the mice in Groups 1 and 2 do not support the suggestion of Price et al. (5) that metastases are mainly established while the primary tumor is still very small. (d) Greater age did not increase the risk of developing metastases from mammary tumors. On the contrary, a general relationship between earlier development and greater malignancy was observed among C3H/He and C3Hf/He mouse mammary tumors.

A considerable proportion of C3H/He mammary carcinomas have the potential to produce metastases. Assuming that only 1 tumor of the average 1.8 tumors per mouse in Group 4A, Table 2, had produced the metastases found at autopsy, it follows that 37% (67%/1.8 tumors) of all large tumors produced metastases. This is in close agreement with the 43% incidence of spontaneous metastases found by Pitelka et al. (4) in 160 retired breeding mice of 7 strains that were carrying large spontaneous mammary tumors. The metastasizing potential of the tumors was, however, not fulfilled in the surgically cured mice (Table 1). Metastases were observed in only 1% (18%/17.3 tumors) of the tumors removed with an average diameter of 5.3 mm; in only 1.5% (22%/15 tumors) of the tumors removed at an average of 9.1 mm; and in only 5.3% (51%/9.7 tumors) of the tumors removed at an average of 11.8 mm. Even if it should not be quite correct to assume that the metastases found in a mouse cured of multiple tumors came from only one of the tumors, the trend from 18% incidence in Group 1A to 67% incidence in Group 4A is still clear. It is important to recognize that the average quantitative values of the metastases found in the 4 C3H/He groups were not significantly different and that metastases did develop from some small primary tumors. This suggests that while the large size of a tumor is not the single dominant factor in the dissemination of cancer cells, a large tumor burden is known to be an important factor in the neutralization of both immune (11) and natural (12) resistance to the development of metastases from mammary tumors. In other words, a mouse that is kept healthy by early surgical cures of its tumors can continue to suppress the development of potential metastases. A second possible mechanism of metastasis promotion is that the higher incidence of metastases in mice with large tumors could be due to growth-promoting tumor products known to be produced by a proportion of C3H/He mammary carcinomas (13).

The question remains whether host immune factors influence
the metastatic process and whether nonimmunogenic tumors are therefore more metastatic. The lack of relationship between the immunogenicity and the metastasizing potential of the tumors in Table 3 suggests that an immune response against mammary tumors may have little inhibiting effect on the development of spontaneous metastases under normal circumstances. (This excludes information influenced by preimmunization or experimental immuno-suppression.) One reason why neoplastic metastasis may show little relationship to tumor immunogenicity may be the fact that effective primary systemic immunity develops slowly even against strongly immunogenic mammary tumors (14, 15) and may not develop in time to prevent the establishment of disseminated tumor cells.

Since the microcinematographic studies of the initial stages of the metastatic process in rabbit ear chambers by Wood et al. (16) in 1961, destruction of the basement membrane and early extravasation after intravascular arrest has been considered an essential step in metastatic tumor growth (17–20). The concept of extravasation has significantly influenced research on the metastatic process, particularly the interest in fibrin and embolic tumor cell arrest and the interest in lytic enzymes produced by neoplastic cells. However, in 1967, Fisher and Fisher (21) suggested that the importance of these factors in the metastatic process may have been exaggerated. Wallace et al. (22, 23) showed that intravascular growth was typical for the Walker 256 carcinoma. Dingemans and Mooi (24) studied tissue invasion in 43 cases of human bronchogenic carcinoma without observing any evidence of destruction of alveolar epithelial basal lamina at the periphery of the invasive tumors. The present microscopic studies of the pulmonary metastases from 95 mammary adenocarcinomas and 6 mammary sarcomas (Table 2) show that, without exception, the adenocarcinomas grew extensively within pulmonary vessels before extravasation (Fig. 2). On the other hand, pulmonary metastases from the mammary sarcomas were always found outside blood vessels (Fig. 3). Active extravasation of the sarcomas probably took place soon after arrest, because intravascular growth of a metastatic sarcoma was never seen. This clear distinction (P < 0.0001 by Fisher’s exact test) in the manner of establishment of metastases from 2 different morphological types of mouse mammary tumors may reflect that the histiocyte or fibrobyte precursors of mammary sarcomas have natural invasive capacities, while the epithelial cell precursors of mammary adenocarcinomas do not.

REFERENCES


15. Price, J. E., Carr, D., and Tarin, D. Spontaneous and induced metastasis of mammary tumors (14, 15) and may not develop in time to prevent the establishment of disseminated tumor cells.


Spontaneous Metastasis from Primary C3H Mouse Mammary Tumors

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