Patterns of Spontaneous Metastasis of Transplantable Hepatocellular Carcinomas

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ABSTRACT

A number of transplantable rat hepatocellular carcinomas of varied phenotype were examined for their ability to metastasize. A striking diversity of pattern was observed, ranging from presentation in almost every organ to none at all. No relationship between metastatic capability and growth rate, tumor size, chromosome composition, or other functional characteristics was noted. Interestingly, several of the tumors demonstrated a lymphatic to vascular route of spread, very similar to that of human tumors.

INTRODUCTION

The major objective of the experimental study of metastasis has been to seek structural, biological, and biochemical characteristics of malignant cells that might correlate with their metastatic potential (1, 8, 10, 14, 22, 37). For the accomplishment of this purpose, a wide variety of spontaneous and induced animal tumors have been examined. In many of these experiments, tumors that were apparently composed of heterogeneous subpopulations of cells have been selected for variant lines with a high degree of metastatic potential or virtually none at all (9, 18). In other instances, tumors that possess a high degree of immunogenicity were induced and utilized to make easier the study of the effect of the immunological responses of the host upon tumor spread (11, 17, 19, 24, 26, 31), this despite the generally accepted finding that the majority of human tumors may be at best weakly immunogenic.

Among the problems in the study of such animal models is the difficulty of producing cancers with spread patterns that are strongly similar to that of human tumor spread. For example, it has been rare for rodent tumors to metastasize by the lymph drainage route, in comparison to the frequency with which human tumors make use of this pathway; it has been even more difficult to locate those that utilize both this and hematogenous spread (27).

Despite the rarity of reports of their use in studies of metastasis (30), it would appear that THC's are a potentially excellent tumor model that has not yet been appropriately exploited. A number of advantages offered by these tumors strongly suggest that such an evaluation would be valuable. The THC of rats have been characterized by a vast array of modalities during their use as a model experimental tumor system (3, 6, 7, 21, 23, 28, 29, 34, 35). They demonstrate a remarkable diversity of phenotypic patterns, which should make possible a multifaceted attempt at correlation with metastatic activity. Further, these tumors were induced and transplanted in inbred rats and have not been deliberately manipulated to alter their metastatic capacity. Lastly, THC are classic tumors for the production of biological markers, such as AFP, and isoenzymes, such as alkaline phosphatase, which may provide a mechanism for a chemical definition of the presence of metastatic foci (25).

The objectives of the experiments reported herein were as follows: (a) to select THC of the rat, which differed distinctively in a number of biological characteristics, and to examine these tumors as a potentially new model for studies of metastasis; (b) to determine whether 1 or any combination of phenotypic patterns is relevant to metastatic potential.

MATERIALS AND METHODS

Female ACI and male and female Buffalo rats (Simonsen Laboratories, Gilroy, Calif.) were obtained at 125 g body weight and were used for transplantation at approximately 150 g. The primary hepatocellular carcinomas from which these THC were derived were induced, respectively, in male ACI and female and male Buffalo rats (6, 21). THC 252, 252b, 251c, and 311c were induced in ACI rats by N-2-fluorenylphthalamic acid. The syn
geneic tumors were transplanted in rats of the opposite sex, in order to aid in identifying the origin of cells isolated from tumors in which chromosomal composition had been determined. Numerous experiments have demonstrated that the characteristics of the tumor cells, including metastatic potential, were not altered by the sex of the recipient.

The THC were implanted s.c. in the midflank. Although several of these tumors had been carried previously by i.m. injection in the hind leg, they grow just as readily s.c. The site of prior transplantation had no influence on the biological behavior of the tumor. Each tumor, which was previously grown i.m., was transplanted s.c. through at least 3 generations prior to the study. Tumor transplantations were carried out as follows. Viable tumor tissue was dissected free from connective tissue and hemorrhagic and necrotic areas under Hanks' balanced salt solution at room temperature. The tumor tissue was transferred to a second aliquot of Hanks' balanced salt solution and was minced as finely as possible with scalpels. The resultant fragments, which usually measured 1 mm or less in diameter, were rinsed once in 0.9% NaCl solution. Under light ether anesthesia and with a 1 ml syringe and a no. 20 disposable
needle, 0.25 ml of this fine brei was injected s.c. into an area that had been previously shaved and cleaned with 70% ethanol. The validity of using the tumor brei and the injection technique was supported by a consistent incidence of tumor take and a constant growth rate for every tumor over many generations.

For i.v. injections the femoral vein was exposed, and 0.25 ml of this brei was injected over a 1-min period. No apparent morbidity or mortality resulted from this procedure.

Complete autopsies were performed on every rat, and sections from each major organ were fixed in neutral buffered formalin. Multiple sections of each lung were obtained. Axillary and inguinal areas from the ipsilateral side were fixed.

RESULTS

Characteristics of THC (Table 1)

The characteristics of THC that have proven to be distinctive and informative in following consecutive transplant generations have been growth rate, chromosomal composition, and AFP production. Throughout the transplant generations used in the current experiments, which ranged from 31 generations for THC 252 to 16 generations for THC 7800, these characteristics have remained constant in tumors of both strains. No significant differences in the results could be attributed either to the strain of rat or to the chemical agent used to induce the tumors.

The histological appearance of each tumor line was identical with that previously reported (6, 21). The histological appearance of the tumor in its metastatic foci was either identical with or similar to that of the transplant and was often sufficiently distinctive to identify the tumor source. Of some interest was the frequent finding of tumor growths entirely within the vessels of the lungs; these apparently were pulmonary veins or alveolar septal capillaries. In several instances, serial sections revealed no extra-vascular components, a finding that has also been described for human liver tumors (4).

The growth rate was defined as the time required from transplantation until a tumor diameter of 3 cm, measured in 2 planes, was achieved. Growth was rapid in 5 of 6 tumors ranging from 2.5 to 3.5 weeks. Only THC 7800 could be considered to be in the medium growth rate of 7 to 8 weeks. Five of the 6 THC were diploid or in the near-diploid range, with 251c being subtriploid. THC 252b, which arose as a subline at the 16th generation of 252, had a remarkable display of 9 stable marker chromosomes (7), while THC 7777 has a unique marker chromosome in approximately 63% of its cells (33).

Transplantation Studies

Sacrifice at 3-cm Diameter (Table 2). For determination of the metastatic potential of THC during a given period of growth, each tumor was transplanted unilaterally into 6 rats, which were sacrificed when the external diameter of the transplants reached 3 cm. In a typical experiment, this ranged from 15 days for 252 to 53 days for 7800. A diverse pattern of metastases was evident, which was independent of growth rate or any other phenotypic characteristic. This diversity was exemplified by the extensive nodal and lung involvement for THC 252 and the total absence of metastasis in THC 311c. In this and all subsequent experiments, lung metastases were of hematogenous (embolic) type; whether resulting from spontaneous metastasis or i.v. injection. Nodal metastases were of the classic sinus-invasive-destructive type.

Excision of THC and Involved Nodes (Table 3). For determination of the temporal sequence of metastasis and the effect of a longer period of observation, each tumor was transplanted unilaterally into 6 rats. Those THC that had demonstrated metastasis at 3-cm diameter, i.e., 252, 252b, and 7777 (Table 2), were now excised at 1 or 2 cm. Those that had not demonstrated metastasis, i.e., THC 251c, 311c, and 7800, were once again allowed to achieve a diameter of 3 cm after which they were excised. Some internal standardization to the time of sacrifice was introduced with 2 factors. Axillary nodal involvement was determined by weekly palpation and was usually detected when the node reached an approximate diameter of 0.5 cm. When positive nodes were thus identified they were excised, and the rat was sacrificed at a time equal to twice the period required for the appearance of nodes. For example, enlarged nodes were apparent at the time of excision of 252b (2-cm diameter) which was at approximately 15 days after transplantation. The rats were sacrificed 30 days later. Following excision of 252b at a diameter of 1 cm, nodes were detected at approximately 24 days and sacrifice took place 48 days later.

Rats that failed to demonstrate enlarged nodes were sacrificed at a time equal to twice the period required to reach the tumor diameter selected for excision. For example, rats bearing THC 7800 were sacrificed at 153 days after tumor transplantation, which was 102 days after excision at 3 cm and 51 days after transplantation.

Despite the extended period of observation prior to sacrifice, only 1 alteration in the pattern of metastasis oc-
Excision. Microscopic or gross metastases were revealed tissue and nodes by careful dissection. Histological examination, if possible, of the route of metastasis of THC 252, the tumor that resulted from i.v. injection, 0.25 ml of THC demonstrated microscopic involvement of at least 1 node in 3 of 6 rats. The animals were sacrificed 1 month following excision. Thus, 251c, which still failed to demonstrate any nodal involvement, now demonstrated multiple lung foci in the lung, demonstrated massive growth bilaterally in all lobes. THC 7777, which had not metastasized to lung, now demonstrated 100% tumor take. THC 311c and 7800 failed to demonstrate any growth in lungs.

**DISCUSSION**

It appears from the results of these experiments that transplantable hepatocellular carcinomas of inbred rats are an interesting and potentially important model for the study of the mechanisms of cancer metastasis. The major purpose of these experiments was to test a relatively small number of available manipulations of these tumors in order to determine and demonstrate some of their diversity in metastatic potential. In experiments not included, alterations in the eventual size of the tumor at death, the site of implantation, or multiplicity of tumor implants frequently influenced the final metastatic pattern. Indeed, one of the intriguing potentials of the use of THC is the availability of tumors with an almost infinite variety of phenotype making feasible a search for properties related to metastases in positive or negative modes.

Although these tumors were not manipulated to select for metastatic capacity, they demonstrated the broadest possible diversity for this activity. Thus, they may metastasize rapidly and widely, either selectively to a single organ or not at all. This individuality is exemplified by THC 252, which spreads to regional nodes, lungs, and many other organs, and by its subline, 252b, which rarely spreads beyond the regional nodes. In this regard, 252 is very similar to human hepatocellular carcinomas (4). It has been suggested previously that the process of transplantation may itself be selective for variation in the ability to metastasize (16). The difference between 252 and 252b could be ant fragments in 0.9% NaCl solution for injection. To determine whether these tumor fragments successfully embolized to the lungs of the recipient rats, we sacrificed 2 rats that had been similarly challenged 30 min after injection. Histological sections revealed many tumor emboli in lung vessels that were composed of cells that appeared histologically viable. Rats that did not die as a result of tumor growth were sacrificed at a time equal to that normally required to achieve a standard transplant diameter of 3 cm. THC 252 and 251c, which had previously demonstrated metastatic foci in the lung, demonstrated massive growth bilaterally in all lobes. THC 7777, which had not metastasized to lung, now demonstrated 100% tumor take. THC 311c and 7800 failed to demonstrate any growth in lungs.

**Table 2**

<table>
<thead>
<tr>
<th>Metastases from THC at 3-cm diameter</th>
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<tbody>
<tr>
<td>Each rat was sacrificed when the THC achieved an external diameter of 3 cm (see Table 1). The numbers in parentheses indicate the number of rats in which the findings were made. Each group includes 6 rats. The 0 to + rating was made as a subjective estimate of total tumor mass. In 4+ lungs every lobe was extensively involved.</td>
</tr>
<tr>
<td>252</td>
</tr>
<tr>
<td>---</td>
</tr>
<tr>
<td>Axillary nodes</td>
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<tr>
<td>Inguinal nodes</td>
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<tr>
<td>Lungs</td>
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<td>Other sites</td>
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<td>Mesentery</td>
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<td>Kidney</td>
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<td>Thymus</td>
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* The location is indicated if 1 or more rats are involved.

**Table 3**

<table>
<thead>
<tr>
<th>Metastases from THC with varying times of sacrifice</th>
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<tbody>
<tr>
<td>Nodal metastasis</td>
</tr>
<tr>
<td>-------------------</td>
</tr>
<tr>
<td>252</td>
</tr>
<tr>
<td>2 cm* (3)</td>
</tr>
<tr>
<td>1 cm (3)</td>
</tr>
<tr>
<td>252b</td>
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<tr>
<td>2 cm (3)</td>
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<td>1 cm (3)</td>
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<tr>
<td>251c</td>
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<tr>
<td>3 cm (6)</td>
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<tr>
<td>311c</td>
</tr>
<tr>
<td>3 cm (6)</td>
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<tr>
<td>7777</td>
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<tr>
<td>2 cm (3)</td>
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<tr>
<td>1 cm (3)</td>
</tr>
<tr>
<td>7800</td>
</tr>
<tr>
<td>3 cm (6)</td>
</tr>
</tbody>
</table>

* Size of tumor at excision.

* Numbers in parentheses, number of rats tested.

* Number of rats positive/total number of rats.

Effect of "Prophylactic" Node Dissection. For determination, if possible, of the route of metastasis of THC 252, the transplants in 6 rats were excised at a diameter of 1 cm. Concurrently, the axillary space was cleared of areolar tissue and nodes by careful dissection. Histological examination of the 2 to 4 identifiable nodes from each rat demonstrated microscopic involvement of at least 1 node in 3 of 6 rats. The animals were sacrificed 1 month following excision. Microscopic or gross metastases were revealed in 4 of 6 rats, 3 of which had also demonstrated nodal involvement.

i.v. Injection (Table 4). For determination of the growth of tumor that resulted from i.v. injection, 0.25 ml of THC brei (approximately 0.1 to 0.15 ml of solid tumor) was injected into the femoral vein. The brei was the result of 2 minces in 0.9% NaCl solution and suspension of the resultants.
considered as an example of selection which, in this instance, apparently resulted from a sudden and major reorganization of the chromosomal composition of the malignant cells (7). Significant alterations of this type, however, are extremely rare in the THC that have been followed for many generations. Further, the major functional characteristics of these tumors have been shown to be remarkably stable and often identical with those of the THC from which they were derived. In the 2.5 years during which these experiments were performed, representing as many as 30 generations for some of the tumors, no alteration in metastatic pattern occurred. These findings suggest that the metastatic patterns of most THC are also stable and are possibly related to those of their tumor of origin. However, it is equally possible that selective pressures of transplantation have resulted in a tumor population of relatively focal character.

The manipulations of these tumors, which have been used to characterize them as a model system, have also resulted in experimental findings that cast doubt upon a number of previously reported concepts of tumor metastasis. These include a correlation between metastatic spread and the tumor-inducing carcinogenic agent (20); the rate of tumor growth and its size (1, 15, 36, 39); the cell karyotype; the state of "differentiation" (10, 32); and immunogenicity (11, 17, 19, 24, 26, 31). Except for the latter, it would appear that the results reported herein strongly limit the possibility that any of these factors is the sole determinant of the process. Thus, tumors induced with a single carcinogen or a closely related analog, of equivalent growth rate and size, roughly comparable chromosomal composition, and expressive of widely varying quantities of AFP have totally differing metastatic capabilities.

The possible intervention of a varying immunogenicity remains unclear since it would require determination for each tumor. It has been reported previously (2) that the primary hepatocellular carcinomas and THC induced by \( N \)-2-fluorenylacetonitrile demonstrate little or no immunogenicity. However, a recent and particularly applicable study has indicated that the immunogenicity of certain Morris THC may be quite varied (30). This conclusion is based upon a partial protection against subsequent tumor growth which was achieved by prior immunization with 1 THC. Thus, without appropriate testing of each of the THC used in this study, no conclusion concerning the role of the immunological response of the host can be offered.

Each of the THC used in this study has also been tested for viral antigens as part of a separate project (C. Sherr and F. F. Becker, unpublished data). At present, no correlation between the presence of such antigens and the metastatic capability of the tumor has been detected.

Of further interest is the frequent use of a lymphatic route of spread by several of these tumors; this route is in many respects strikingly similar to that of human malignant disease (4, 10, 26, 32). Indeed, several THC are available that fulfill "all" of the criteria of a suitable animal model for the study of metastasis (27) and that include their transplantation in a syngeneic host, consistency of pattern over many generations, measurable antigenicity; spread by comparable routes, and perhaps even their response to known therapeutic modalities (5). The heterogeneous pattern of spread by these various tumors is strongly reminiscent of that demonstrated by clones of cells obtained from parent lines of mouse melanoma (12).

Although a number of factors have been proposed as possibly influencing the ability of the malignant cell to metastasize, as well as affecting the sites at which secondary growth will occur, metastatic potential basically is dependent upon either the properties of the malignant cells themselves or the defenses of the host. For example, malignant cells could possess surface alterations that would enable them to evade recognition and destruction by the host. In this instance, spread would be limited only by the invasiveness of the cell and its ability to acquire support for growth elsewhere. Primary host defenses could be classified as local or systemic. Cellular mechanisms inherent in tissues, such as in regional nodes (13, 37, 38), dominate the former; while systemic defenses are considered to include the entire spectrum of immunological mechanisms and other factors such as opsonins (15). An example of the possible interaction between local host defenses and tumor take might be found in 252b. Thus, the suggested presence of particularly destructive factors in the lungs (27) would correlate with the failure of 252b to appear as a pulmonary metastasis, despite massive invasion and destruction of axillary nodes, and the scarcity of pulmonary foci with i.v. administration.

At present, modification of the defense capacity of the host by the malignant cell is considered to play a crucial role in the pathogenesis of metastasis. Preliminary experiments have indicated that 311c, when planted in the muscles of both hind legs, may be capable of pulmonary metastases, despite its failure to produce such metastases when injected directly into the femoral vein. It is possible that the combination of a propensity to venous embolization from this site superimposed on a substantial tumor burden might explain this dichotomy. Tumor burden has been malignant to alter significantly the response of the host to malignant cells (10, 15, 37).

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REFERENCES


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