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# Characterization of Growth Properties and Demonstration of the Tumor-specific Transplantation Antigens of Morris Hepatomas[1]

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## SUMMARY

The growth properties of single-tumor-cell suspensions prepared by enzymatic digestion of solid tumors from Morris hepatomas 7777, 5123tc, and 3924a and the presence of tumor-specific transplantation antigen for tumor lines 7777 and 3924a were described. Two of the tumor cell lines (7777 and 3924a) showed consistent i.m. tumor growth following the inoculation of $1 \times 10^5$ tumor cells, and a similar dose of 5123tc tumor cells resulted in inconsistent tumor growth. Two of the tumor lines (5123tc and 7777) were associated with rapid appearance of lung metastases, whereas with line 3924a metastatic lung lesions rarely developed despite its rapid i.m. tumor growth rate.

Tumor resistance to rechallenge with a threshold inoculum of tumor cells was present in approximately 15 to 50% of the animals following amputation of an existing tumor mass. Resistance to a challenge tumor cell inoculum could also be accomplished by immunization with irradiated tumor cells. Tumor-specific resistance was demonstrated to tumor line 3924a in that "immune" animals were able to resist a challenge with 3924a tumor cells but did not resist a challenge with tumor line 9098.

## INTRODUCTION

Previous studies have reported the biological characteristics of transplantable Morris hepatomas. The largest majority of these studies have dealt with the interesting biochemical aspects of these tumors. We have studied these tumors to delineate the relationship that tumor growth has to production of $\alpha$-1-fetoprotein (6, 12, 14), and we are currently using them for studies in tumor immunology and biology.

The growth characteristics of some of these tumors have been investigated by others, but the majority of the previous reports have been performed with mechanical cell suspensions or minced tumor preparations. No studies have reported the growth parameters of a single-cell suspension of tumor cells prepared by enzymatic digestion of a solid i.m. tumor. These studies were necessary background information needed to design experiments to determine whether tumor-specific antigens existed in these hepatic tumors.

Tumors induced by different carcinogens vary markedly in antigenicity, and these antigenic differences may be quantitative or qualitative in nature. Numerous studies have been performed in many animal species, but only a few have been done in inbred rats. The majority of the studies have been conducted with highly antigenic sarcomas induced by methylcholanthrene or epitheliomas induced by polycyclic hydrocarbon carcinogens. Neoplasms induced by other chemical carcinogens have received significantly less attention.

In this report we summarize information about the growth properties of 3 Morris hepatoma tumor cell lines (7777, 5123tc, and 3924a). We also present information that demonstrates that tumor antigens are present in transplantable tumor cell lines (7777, 3924a).

## MATERIALS AND METHODS

### Animals

Buffalo inbred male rats (13 weeks old) (250 g) and ACI inbred male rats were purchased from Simonson Labs, Inc., Gilroy, Calif. They were housed in groups of 4 in stainless steel boxes with stainless grid tops and were supplied sawdust bedding, fresh water, and Purina laboratory rat chow *ad libitum*.

### Tumors

All of the transplantable hepatomas described in this paper were originally obtained from Dr. Harold P. Morris of the Department of Biochemistry, Howard University College of Medicine, Washington, D. C. Tumor lines 7777 and 5123tc were maintained in inbred Buffalo male rats and tumor lines 3924a and 9098 were maintained in inbred ACI male rats.

Tumor line 5123tc is a tissue culture variant of a moderately differentiated trabecular hepatocellular carcinoma induced after the dietary administration of FPA.[3] The tumor was received from Dr. Morris in the 106th transplant generation. In this study transplant generations 106 to 120 were used.

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[3] The abbreviations used are: FPA, *N*-2-fluorenylphthalmic acid, FdiAA, *N*-2-fluorenyldiacetamide; MEM, Eagle's minimal essential medium; i.d., intradermal.

Tumor line 7777 is a poorly differentiated hepatocellular carcinoma induced after the dietary administration of FPA. It was received from Dr. Morris in the 85th transplant generation, and transplant generations 85 to 95 were used in this study.

Tumor line 3924a is a poorly differentiated hepatocellular carcinoma induced after the dietary administration of FdiAA. It was received from Dr. Morris in the 299th transplant generation, and transplant generations 300 to 310 were used in this study.

Tumor line 9098 is a poorly differentiated hepatocellular carcinoma induced by the dietary administration of FdiAA. It was received from Dr. Morris in the 86th transplant generation, and transplant generation 96 was used in this study.

### Tumor Cell Preparation

Tumor-bearing animals were anesthetized by an i.p. injection of sodium pentobarbital (40 mg/kg). The i.m. tumor was surgically removed, and the tumor was dissected from the surrounding muscle and connective tissue. The tumor tissue was weighed, minced in a plastic Petri dish (Falcon Plastics Co., Los Angeles, Calif., 1001 Optilux) containing a small amount of MEM (Grand Island Biological Co., Grand Island, N. Y.). The minced fragments of tissue were resuspended in Erlenmeyer flasks containing MEM and 0.25% trypsin-EDTA (Grand Island Biological Co.) in MEM, 5 ml of media per g of tumor tissue, and mixed for 5 min at room temperature. The cell-rich supernatant was then decanted through 2 layers of clinical gauze in 50-ml graduated plastic conical centrifuge tubes (Falcon Plastics Co. 2070 tube) and centrifuged at 100 × *g* for 10 min. The cells were resuspended in double the volume of medium and stored at room temperature. To obtain additional cells, the same mixing, centrifuging, and resuspending procedures were performed on the tumor tissue with fresh MEM and 0.25% trypsin-EDTA with mixing for 10 min. This above sequence is repeated twice. A pool was made of tumor cells obtained from the latter 2 or 3 tumor cell digestions as these preparations most frequently contained the largest number of viable cells. A viability count was done with 0.1% trypan blue as previously described, and cell preparations contained 80 to 90% viable cells (12).

### Methods of Immunization

**Surgical Excision of Tumor.** Tumor masses were excised from either i.d. or i.m. sites using routine surgical procedures. Animals were anesthesized by the i.p. injection of pentobarbital (40 mg/kg). The surgical field was prepared in a routine antiseptic fashion, the skin incision was made, and the location of the tumor was noted to avoid excision through the tumor tissue. Distal venous and arterial vessels were ligated, and the tumor and adjacent soft tissue margin were excised. The wound was closed with 4-0 silk suture, and autoclips were used to close the skin incision.

**Immunization with Irradiated Tumor Cells.** A single cell suspension of tumor cells was prepared as described above. The tumor cells were placed in a glass culture tube (14 x 100 mm) and were given a dose of 10,000 rads from a $^{60}$Co irradiation source. Animals were given i.m. injections into the gastrocnemius muscle, $5 \times 10^6$ tumor cells being injected into each rear leg. The injections were given at biweekly intervals.

**Challenge of Immunized Animals.** Tumor challenge was done using a suspension of tumor cells prepared as previously described. If an animal had been amputated, he was given an injection in the contralateral limb. If he had been immunized using irradiated tumor cells, he subsequently was challenged in the right rear leg.

## RESULTS

### Characterization of Growth Properties

**Tumor Growth i.m.** A summary of the i.m. dose titrations performed with the 3 tumor cell lines is presented in Table 1. Tumor growth occurred with all of the tumor lines examined when $1 \times 10^6$ tumor cells were inoculated, but the level at which less than 100% tumor growth occurred varied. With tumor cell line 5123tc, a cell inoculum of $1 \times 10^5$ resulted in growth in only 9 of 11 (82%) of the animals, whereas with tumor cell line 3924a $1 \times 10^5$ tumor cells gave 100% takes but $1 \times 10^4$ tumor cells caused tumor growth in only 4 of 8 (50%) animals. A cell inoculum of as few as 100 cells could occasionally be associated with tumor growth in 1 of 13 (8%). The metastatic pattern of these tumor cell lines differs in that with the i.m. tumor growth of 5123tc and 7777, large numbers of lung metastases occurred in the lungs, whereas with tumor cell line 3924a metastatic lung lesions were infrequently noted and then only in association with a large i.m. tumor.

**i.d., Intrafootpad, and i.p. Tumor Growth.** The results of tumor growth at various sites of inoculation are summarized in Table 2. Tumor growth i.d. varied but was most consistently observed with tumor line 3924a where 6 of 7 animals grew i.d. tumors. Intrafootpad tumor growth was observed in all of the inoculated animals, but compared with a similar cell inoculum in the i.m. site the appearance of palpable tumors was significantly delayed. As an illustration of this, with tumor line 7777 an i.m. tumor cell inoculum of $1 \times 10^6$ was palpable in all animals by Day 12 but a 3-fold higher inoculum intrafootpad did not cause palpable tumors in all animals until Day 37. A bloody ascites tumor cell line was successfully established with tumor cell line 3924a, and it has been successfully maintained over 10 generations with a threshold dose of approximately 10 million cells. Several attempts have been made to establish ascites tumor cell lines from 7777 and 5123tc, but all have been unsuccessful and tumor growth is observed only in the mesenteric fat.

**Demonstration of Tumor-specific Transplantation Antigens.** An attempt was made to demonstrate the existence of tumor antigen by the temporary growth and excision method or by immunization with X-irradiated tumor cells. The results are summarized in Table 3. With tumor line 7777 a group of 8 animals that had been given injections of $8 \times 10^6$ tumor cells i.m. had their tumors amputated on Day 15, and 2 weeks postamputation they were challenged with $1 \times 10^5$ tumor cells. Of the amputated animals 3 of 8 resisted subsequent tumor challenge, but 2 of 3 "resistant" animals had a regrowth of the original tumor at the site of amputa-

Table 1

*I.m. dose titration of single-cell suspension of tumor cells from tumor lines 5123tc, 3924A, and 7777*

| Tumor line | Experiment | Dose of tumor cells inoculated[a] | | | | | |
|---|---|---|---|---|---|---|---|
| | | $10 \times 10^6$ | $1 \times 10^6$ | $1 \times 10^5$ | $1 \times 10^4$ | $1 \times 10^3$ | $1 \times 10^2$ |
| 5123tc | 1 | 2/2[a] | 6/6 | 5/6 | 1/3 | | |
| | 2 | | 5/5 | 4/5 | 0/5 | 0/5 (0/6)[b] | |
| 3924A | 1 | 4/4 | 4/4 | 4/4 | 3/4 | | |
| | 2 | 2/2 | 3/3 | 3/3 | 1/4 | 1/3 | 0/3 |
| | 3 | 7/7 | 4/4 | 7/7 | | 0/8 | |
| 7777 | 1 | 5/5 | 11/11 | | | | |
| | 2 | 2/2 | 2/2 | 5/5 | 5/5 | 1/5 (0/8) | 0/5 |

[a] Results expressed as the number of animals with tumor per number of animals inoculated.
[b] Results in parentheses are from a separate experiment.

Table 2

*Growth characteristics of tumor lines 7777, 3924a, and 5123tc*

| Tumor line | Site of tumor inoculation | | Ascites line development ($50 \times 10^6$) |
|---|---|---|---|
| | i.d.[a] ($3 \times 10^6$) | Intrafootpad ($3 \times 10^6$) | |
| 7777 | 2/6 | 6/6 (10/10) | Not successful, died 20 days, tumor in mesenteric fat |
| 3924a | 6/7 | 3/3 (10/10) | Successful i.p. variant established |
| 5123tc | 3/6 | 6/6 | Not successful, tumor grew in peritoneal fat |

[a] Results expressed as the number of animals with tumor per number of animals inoculated.

Table 3

*Immunization of animals against Morris hepatoma 7777 and 3924a*

| Method of immunization | Tumor line 7777[a] | Tumor line 3924[a] |
|---|---|---|
| Tumor growth i.m. followed by amputation | 5/8[b] | 3/6 |
| Controls | 8/8 | 8/8 |
| Immunization by the i.m. inoculation of X-irradiated tumor cells[c] | 0/6 (0/6)[d] | 0/6 |
| Controls | 5/7 (6/6) | 3/3 |

[a] Results expressed as number of animals with tumor per number of animals challenged.
[b] Two of the animals that resisted challenge had recurrence of their tumor at the site of amputation.
[c] Injected 3 times with $10 \times 10^6$ tumor cells.
[d] Since the control animals did not all develop tumor, the "immune" animals were injected twice more with $10 \times 10^6$ irradiated cells and rechallenged with a new control group.

tion. We postulated that this resistance might be secondary to a high level of concomitant immunity present in the tumor-bearing animals. To investigate this, we rechallenged all of the remaining animals in both the experimental and control groups with $1 \times 10^5$ tumor cells. We observed the following: (*a*) of the 5 amputated animals in which the 1st challenge inoculum grew, the 2nd tumor cell inoculum grew in only 2 of the animals; (*b*) the one totally resistant amputated animal again resisted rechallenge; (*c*) the 2nd challenge inoculum grew in 5 of 8 of the control animals; and (*d*) challenge inoculum grew in 5 of 6 of a new control group of animals. Because of the inconsistent growth in the second control group, these results are only suggestive that concomitant immunity existed.

A similar amputation-rechallenge experiment was performed with tumor line 3924a. Preliminary experiments had demonstrated tumor resistance in 2 of 4 animals that had i.d. tumors amputated. We inoculated 6 animals i.m. with $10 \times 10^6$ tumor cells, amputated their tumor on Day 17, and rechallenged them with $1 \times 10^5$ tumor cells on Day 17. Of the amputated animals, 3 of 6 resisted tumor challenge whereas none of the control animals resisted tumor challenge.

Immunization with multiple doses of X-irradiated tumor cells was generally more effective for the induction of tumor immunity than was excision of an established tumor mass. With both tumor line 7777 and 3924a, all animals were rechallenged with $1 \times 10^5$ tumor cells 10 days following the last immunization inoculum of "X-irradiated tumor cells." With tumor line 7777 the 1st challenge grew in only 5 of 7 of the control animals, and no growth occurred in the "immune" animals. Because of this we elected to boost the immune animals by giving them 2 injections of $10 \times 10^6$ X-irradiated tumor cells and then rechallenging them. In the 2nd rechallenge tumor growth occurred in all of the controls and in none of the immunized animals.

Tumor resistance was also noted in animals that were immunized with 3924a X-irradiated tumor cells in that all of

the immunized animals resisted challenge with $1 \times 10^5$ tumor cells. We had observed that the i.m. tumor growth of tumor line 3924a was rarely associated with pulmonary metastases. From this observation we suspected that this tumor line was highly immunogenic. To test this hypothesis we challenged the immune animals with $1 \times 10^6$ tumor cells 10 days after the 4th immunizing inoculation of $10 \times 10^6$ tumor cells. This dose was 10 times above the threshold dose of $1 \times 10^5$. Tumor growth occurred in all animals with no difference in growth rate being observed between the immune animals and the control group, suggesting that this is not a highly antigenic tumor line.

The hypothesis that tumor immunity existed to tumor line 3924a is supported by other observations. On rare occasions animals that were given i.p. injections of the ascites tumor cells would not develop tumors. Of 7 animals that were "survivors" of injections of $50 \times 10^6$ i.p. tumor cells, 3 of the animals subsequently resisted an i.m. challenge with $1 \times 10^6$ tumor cells.

The final experiment with tumor line 3924a was designed to determine whether a group of animals immunized by 5 injections of $10 \times 10^6$ X-irradiated tumor cells would show tumor-specific resistance to a challenge of 3924a tumor cells but not to a different syngeneic tumor cell line 9098. Ten animals were in both the experimental and control groups. All of the animals were inoculated i.m. with $2 \times 10^5$ line 3924a tumor cells in the right rear leg, and they received $2 \times 10^5$ line 9098 tumor cells in the left rear leg. The results are summarized in Table 4. Tumor line 9098 grew in all of the control animals and in all of the 3924a immune animals. The tumor cell inoculum of 3924a grew in all of the control animals, but in only 1 of the 10 immunized animals. The 3924a-immunized animals, therefore, showed some degree of tumor specificity as they did not resist a tumor cell challenge with tumor line 9098.

## DISCUSSION

These studies describe the growth properties of 3 hepatoma tumor cell lines (7777, 5123tc, and 3924a) and the tumor antigenic properties of 2 tumor cell lines (7777 and 3924a). This is not the 1st report that describes the growth properties of Morris hepatomas in inbred rats, but it does present the growth characteristics of a quantitated number of tumor cells that have been prepared following enzymatic digestion of an i.m. tumor mass.

The previous investigators have not described the growth properties of a quantitated number of single tumor cells that had been inoculated into different sites of the body. This information was essential in the design of tumor immunity studies which were being pursued in our laboratories.

Table 4

*Demonstration of tumor-specific immunity to tumor line 3924a*

| | Growth of 3924a tumor[a] | Growth of 9098 tumor[a] |
|---|---|---|
| 3924 "immune" animals[b] | 1/10 | 10/10 |
| Control animals | 10/10 | 10/10 |

[a] Results are expressed as number of animals with tumor per number of animals challenged.

[b] All animals had received a total of 5 i.m. injections with $10 \times 10^6$ X-irradiated tumor cells and had resisted a challenge inoculum of $1 \times 10^5$.

Morris and Wagner (9) using these and many other hepatomas have described the growth rate of hepatomas by correlating the size of the transplantable hepatoma with the time between inoculations. The time between inoculations was the time in days that the tumor grew before being transplanted to a new animal, and the tumor size is calculated by adding together the length and width of the tumor. These measurements have been most useful within their laboratory where tumors of the same size are passaged, and the variables inherent in the measurement of the tumor mass are somewhat standardized. Other investigators have placed emphasis upon defining methods to express the changes in the tumor with time (7, 8, 13). The reported studies are the 1st demonstration of the variable growth rates and patterns of Morris hepatoma tumors following the inoculation of a quantitative number of tumor cells that were obtained from enzymatic digestion of a solid i.m. tumor.

Odashima and Morris (10) have reported that it was possible to establish an ascites hepatoma line from tumor line 3924a. The histology of the i.p. growing tumor nodules, percentage of transplants, survival time in days, and size of tumor cell clusters have been reported (10) and our studies (unpublished data) are in full agreement with these observations. We were not able to establish successfully ascites tumor cell lines from tumor line 7777 and 5123tc.

Studies to investigate the presence of tumor-specific antigen in chemically induced transplantable hepatoma tumors have been conducted in both guinea pigs and rats (1, 3, 4, 15, 16). Hepatomas induced by diethylnitrosamine in inbred guinea pigs vary greatly in their immunogenicity. Some tumor lines were highly immunogenic whereas with others tumor-specific resistance could not be demonstrated using the conventional technique of surgical excision (15, 16). A study in rats suggests that diethylnitrosamine tumors in rats also possessed tumor-specific antigen(s) (3). Rat hepatomas induced by dimethylaminoazobenzene possess tumor-specific transplantation antigens (1, 4). Acetylaminofluorene-induced hepatomas in inbred rats possessed only weak tumor-specific antigens. When immunoprotection was attempted by immunization with irradiated tumor cells, immunoprotection was observed in only 3 of 9 animals; when immunization was effected by surgical removal of tumors, immunoprotection was not observed in any of the 5 animals (3). Similar results were observed with other acetylaminofluorene-induced tumors (2). Our studies have demonstrated the presence of tumor antigen in Morris hepatoma lines 3924a and 7777. Tumor resistance to rechallenge with tumor line 3924a (FdiAA-induced tumor) was induced by the surgical excision of an existing tumor mass or by immunization i.m. with inoculations of X-irradiated tumor cells. Tumor resistance was also present in some animals in which there was a failure of an i.p. inoculum of tumor cells to grow. That this resistance was tumor-specific was demonstrated by the failure of 3924a-immunized animals to resist i.m. tumor challenge with tumor line 9098 (an FdiAA-

induced tumor). The studies performed to demonstrate tumor resistance to tumor line 7777 (FPA-induced tumor) were less conclusive. Surgical excision of an initial tumor mass followed by rechallenge resulted in complete resistance in only 1 of 6 animals, whereas inhibition of tumor growth was observed in 2 animals that had regrowth of their tumor at the site of amputation. Attempts to demonstrate the presence of concomitant immunity were not conclusive and further studies are needed to evaluate this tumor-host relationship. Other investigators have demonstrated that concomitant immunity is generally much weaker than immunity induced by the excision of the tumor, but that on occasion the demonstration of concomitant immunity can be used as a criterion for the existence of tumor-specific antigenicity (11). This has been done even when tumor-specific immunity could not be induced by using X-irradiated tumor cells. Immunization with X-irradiated tumor cells of line 7777 resulted in the failure of a challenge of 7777 tumor cells to grow.

Further studies are being conducted to evaluate whether these hepatoma lines possess embryonic antigens that are similar to those described by Baldwin for the dimethylaminoazobenzene hepatoma tumors (5) as well as tissue-specific antigens.

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