Europ. J. Cancer Vol. 17, pp. 71–76.
© Pergamon Press Ltd. 1981. Printed in Great Britain

0014-2964/81/0101-0071 $02.00/0

Characterization of Tumor Lines Derived from Spontaneous Metastases of a Transplanted Murine Sarcoma*

ALBERTO MANTOVANI, RAFFAELLA GIAVAZZI, GIULIO ALESSANDRI, FEDERICO SPREAFICO and SILVIO GARATTINI

Istituto de Richerche Farmacologiche "Mario Negri", Via Eritrea 62—20157 *Milan, Italy*

Abstract—*Cell lines were obtained from nine individual spontaneous metastases of the murine mFS6 sarcoma by s.c. inoculation of lung deposits in syngeneic mice. When inoculated i.m., two lines (M4 and M7) from metastases had greater, and two (M8 and M9) had less metastasizing capacity than the primary mFS6 sarcoma. The remaining cell lines gave spontaneous metastases similar in incidence, number and weight to the primary mFS6 tumor. When inoculated i.v., M2 and M4 gave consistently more lung deposits than mFS6 cells, whereas cells from M1, M5, M6 and M8 produced fewer artificial metastases than the primary tumor. Eight individual lung metastases were disaggregated and tested for metastasizing capacity immediately after isolation from the lung parenchyma: spontaneous and artificial metastases from metastatic cells were similar to the primary tumor, except for one secondary which was less metastatic. The immunological characteristics of cell lines from metastases were studied by* in vivo *immunization and challenge. Seven lines from metastases and the primary tumor had weak cross-reacting transplantation antigens whereas two lines (M3 and M4) had no demonstrable antigenicity. Thus, cell lines from individual spontaneous metastases are heterogeneous in many respects, including metastasizing capacity. However in this model metastasis does not appear to be the expression of strong selection of variant cells with increased metastatic potential.*

INTRODUCTION

METASTASIS is a crucial event in malignancy but the pathogenesis of cancer cell dissemination and metastasis remains largely to be defined [1–3]. Tumor cell clones from primary murine neoplasms differ dramatically in their metastatic potential [4, 5]. Accordingly, it has been proposed that metastases originate from variant cells with greater metastasizing capacity preexisting within the primary neoplasm, and that metastasis formation is the ultimate expression of a strongly selective multistep process [3–5]. According to this hypothesis, therefore, tumor cellsf from spontaneous metastases should be better able to undergo the multistep process of metastatic dissemination.

Accepted 10 July 1980.

*This work was supported by grant ROI-CA-12764 from the Division of Cancer Treatment, National Cancer Institute, Bethesda, MD, USA, and by grant 78.02792.96 from Consiglio Nazionale delle Richerche, Rome, Italy.

To test this, we obtained tumor cell lines from individual spontaneous pulmonary deposits of a transplanted chemically-induced fibrosarcoma and studied several of their biological properties.

MATERIALS AND METHODS

Mice.

C57Bl/6 mice, 8–10 weeks old, were obtained from Charles River, Calco, Italy.

Tumor lines

The weakly immunogenic, spontaneously metastasizing, benzo[a]pyrene-induced mFS6 sarcoma has been recently described (Mantovani, 1978). The tumor was used in its 5–10th passage generation. The mFS6 sarcoma is weakly immunogenic, 60 and 75% of immunized mice rejecting challenge with 10^4 and 10^3 tumor cells, respectively, and has a macrophage content of 19%; the tumor spontaneously metastasizes to the lung in 45% of

i.m.-injected hosts [6]. Tumors obtained 2–3 weeks after implantation were minced with scissors and disaggregated by exposure to 0.3% trypsin in basal medium Eagle (BME). The cells were washed twice with 50 ml BME and resuspended at a concentration of 10^6 cells/ml BME. The tumor cell suspension (0.1 ml) was injected i.m. in the right hind thigh of syngeneic mice. After 4–5 weeks, when the primary tumors weighed 4–5 g, the mice were killed and the lungs were aseptically removed and examined with a dissecting microscope. Lung secondaries were dissected free of gross lung parenchyma, rinsed with BME and transplanted s.c. with the aid of a trochar into the backs of individual mice. The resulting tumors, each derived from an individual lung nodule and designated M1–M9, were aseptically collected when they weighed 4–6 g, and stored in liquid nitrogen. The tumor lines were used within 2–3 *in vivo* passages, which were made by s.c. transfer of tumor fragments with a trochar. The M1 and M2 lines derived from two mice with a solitary nodule. When multiple metastases were found in lungs, cell lines from two or three individual nodules from the same host were obtained, and these were: M3 and M4; M5, M6 and M7; M8 and M9.

In a series of experiments, the metastasizing capacity of individual spontaneous lung metastases was tested immediately after isolation from the lung parenchyma without prior passage s.c. in syngeneic hosts. Lung deposits (usually $\simeq 3$ mm in diameter) were dissected free of lung parenchyma and disaggregated by exposure to trypsin as described above.

Metastasizing capacity

Mice were injected i.m. in the right hind thigh with 10^4 trypsinized tumor cells in 0.1 ml BME. Tumor diameters were measured twice a week with calipers. At death, the number and weights of spontaneous lung secondaries were measured as previously described [7]. To evaluate artificial (i.v.) metastases, 10^5 tumor cells in 0.5 ml BME were inoculated i.v. and the mice were killed 18 days later.

Immunogenicity

Mice were immunized by surgical excision of the growing tumors 8–12 days after s.c. implantation of 10^4 cells. Controls were sham operated. Ten days after surgery, the mice were inoculated with either a standard i.m. inoculum of 10^4 tumor cells or, in a more limited series of experiments, with graded doses (from 10^2 to 10^6) of cells.

Statistical analysis

Results are representative of at least three experiments. The incidence of mice with metastases over total number of tumor-transplanted animals and the incidence of tumor takes were analysed by Fisher's exact test. Differences in survival time were analysed by the Mann–Whitney *U*-test, and for metastasis weight Duncan's new multiple range test was used.

RESULTS

The capacity of tumor lines to metastasize spontaneously was investigated after i.m. inoculation of 10^4 tumor cells. No consistent differences in the growth of i.m.-inoculated tumor cells were detected as judged from the median survival time of tumor-bearing mice (Table 1) and from tumor diameters measured every other day (results not presented). The primary mFS6 sarcoma and cell lines from metastatic lung deposits were heterogenous in their capacity for giving spontaneous lung nodules (Table 1). Compared to the primary mFS6 tumor, the M4 and M7 cell lines showed a significantly greater number both of mice with spontaneous lung metastases and of lung lesions per animal. Similarly, the weight of lung metastases from the M4 and M7 lines was also greater. In contrast, M8 and M9 tumor cells had little metastatic potential. Cell lines from the remaining nodules (M1, M2, M3, M5 and M6) were not significantly different from the primary mFS6 sarcoma. Spontaneous extrapulmonary metastases were never detected.

To elucidate further the metastasizing capacity of the cell-lines, artificial (i.v.) metastases were studied (Table 2). IV-injected M2 and M4 tumor cells gave consistently more lung nodules than mFS6 cells. Moreover, with M2 and M4 occasional gross neoplastic foci were detected at autopsy in liver and kidney. In contrast, cells from the M1, M5, M6 and M8 lines produced fewer artificial metastases than the primary mFS6 sarcoma. Lung lesions after i.v. inoculation of the M3, M7 and M9 lines were comparable to the primary neoplasm. It is of interest that there was little correlation between artificial and spontaneous metastases from the various cell lines (Tables 1 and 2). For instance, although M4 had more artificial and spontaneous lung lesions than the primary, the M2

Table 1. Spontaneous metastases of tumor cell lines derived from lung secondaries of the mFS6 sarcoma

Tumor line	MST*	Mice with metastases/ total	Metastases number (±S.E.)†	Metastases weight (mg±S.E.)†
Primary mFS6	39 (25–50)	17/32	3.3 (0.3)	18.2 (5.4)
M1	38 (25–49)	4/8	5.2 (3.2)	50.1 (40.9)
M2	33 (28–55)	6/15	3.2 (1.2)	7.8 (7.0)
M3	36 (25–48)	10/16	8.7 (3.0)	48.9 (33.2)
M4	36 (30–49)	13/14‡	16.7 (3.6)‡	122.5 (38.5)‡
M5	33 (25–41)	10/15	8.7 (1.8)	45.7 (20.0)
M6	31 (27–42)	10/15	7.8 (2.9)	11.3 (4.0)
M7	44 (33–52)	15/15‡	13.8 (2.6)‡	170.2 (12.7)‡
M8	35 (26–27)	1/16‡	1.0	0.5
M9	38 (30–51)	0/15‡	—	—

*Median survival time with range shown in parentheses.
†Number and weight of lung metastases/mouse.
‡$P<0.01$ compared to primary mDS6 sarcoma.

Table 2. Artificial (i.v.) metastases of tumor cell lines derived from lung secondaries of the mFS6 sarcoma

Tumor line	Mice with metastases/ total	Metastases number (±S.E.)*	Metastases weight (mg±S.E.)*
Primary mFS6	7/8	34.8 (4.8)	21.2 (5.0)
M1	8/8	3.0 (0.4)†	3.4 (0.9)†
M2	8/8	123.5 (21.9)‡	97.5 (40.5)‡
M3	8/8	39.5 (8.8)	20.5 (5.1)
M4	8/8	83.0 (10.4)‡	43.2 (5.4)
M5	7/8	2.9 (0.9)†	1.5 (0.4)†
M6	8/8	2.5 (0.2)†	1.3 (0.1)†
M7	8/8	49.6 (6.3)	25.7 (3.3)
M8	7/8	6.3 (1.6)†	3.3 (0.8)†
M9	7/8	22.8 (5.0)	13.5 (2.3)

*Number and weight of lung metastases/mouse.
†$P<0.05$ compared to primary mFS6 sarcoma.
‡$P<0.01$ compared to primary mDS6 sarcoma.

line, which was the most efficient in the i.v. assay (Table 2), had spontaneous metastases similar to the primary mFS6 tumor (Table 1). Conversely, M7 was hypermetastatic after i.m., but not after i.v., inoculation.

Results presented in Tables 1 and 2 were obtained with tumor lines derived from metastases by s.c. inoculation in syngeneic mice. Since this procedure may have significantly altered the biological properties of cells from metastases, in a series of experiments individual lung lesions were disaggregated and tested for metastasizing capacity immediately after isolation from the lung parenchyma (Table 3). None of the eight metastatic cell preparations had increased metastatic potential after i.m. inoculation, compared to the primary tumor (Table 3); M18 gave less spontaneous lung metastases than the primary neoplasm. From four lung nodules cells obtained were sufficient for testing also artificial (i.v.) metastases: results were similar to those obtained with i.m. inoculation.

Host defense mechanisms could play a significant, though largely undefined, role in the control of metastases, and tumor cells from lung secondaries reportedly express antigenic specificities different from the primary tumor [8–11]. Therefore we studied the antigenicity of cell lines from individual metastases by *in vivo* immunization and challenge (Table 4). Mice were immunized by surgical excision of the primary mFS6 sarcoma and inoculated with 10^4 cells of the various lines. The 10^4 inoculum, rejected by 47% of the immune mice, was initially used because under these conditions no significant protection against higher mFS6 tumor cell doses (10^5) was observed [6]. Immunization with the mFS6 sarcoma failed to confer significant protection against the M3 and M9 tumor lines whereas

Table 3. Spontaneous metastases from lung secondaries of the mFS6 sarcoma

Tumor cells		MST†	Metastases number (±S.E.)	Metastases weight (mg±S.E.)
Primary tumor		37 (34–41)	13.5 (6.3)	49.0 (23.7)
Metastasis No.	11	42 (35–48)	8.7 (2.6)	26.4 (13.1)
	12	42 (33–48)	7.6 (3.5)	14.1 (7.7)
	13	36 (28–50)	16.6 (5.6)	38.2 (16.3)
	14	35 (30–37)	8.3 (2.7)	7.8 (2.6)
	15	37 (29–39)	9.5 (2.4)	24.8 (15.2)
	16	32 (26–39)	4.4 (1.5)	3.8 (1.1)
	17	37 (32–40)	18.0 (7.5)	48.2 (28.8)
	18	35 (27–37)	2.5 (0.9)‡	5.4 (3.2)

*Individual lung metastases were disaggregated immediately after isolation from the lung parenchyma; 10^4 cells were injected i.m.
†Median survival time with range shown in parentheses.
‡$P<0.05$ compared to the primary tumor.

Table 4. Antigenicity of tumor cell lines derived from lung metastases of the mFS6 sarcoma

Tumor line*	Takes/total Control mice	Immune mice
Primary mFS6	20/20	18/38†
M1	15/16	11/17†
M2	12/12	5/13†
M3	12/12	10/11
M4	25/25	20/31†
M5	15/15	6/15†
M6	20/20	10/23†
M7	12/12	11/20†
M8	11/11	6/11†
M9	15/15	14/17

*Mice were immunized against the primary mFS6 sarcoma and inoculated with either the primary tumor or cell lines from metastases.
†$P<0.05$ compared to control mice.

tumor cells from the other metastatic lines were as antigenic as the primary mSF6 tumor.

In a limited series of experiments small numbers of mice (eight per experimental group) were immunized against the mFS6 sarcoma and given graded numbers of tumor cells; the results confirmed data obtained with a challenge of 10^4 cells, as illustrated by the experiment with M3 and M9 shown in Table 5.

Table 5. Antigenicity of tumor cell lines derived from lung metastases of the mFS6 sarcoma

Tumor line*	Cell number	Takes/total Control mice	Immune mice
Primary mFS6	10^3	10/10	2/8†
	10^4	10/10	3/8†
	10^5	10/10	7/8
M3	10^3	7/7	6/6
	10^4	7/7	4/5
	10^5	7/7	7/7
M9	10^3	8/8	8/8
	10^4	8/8	7/9
	10^5	8/8	8/8

*Mice were immunized against the mFS6 tumor and inoculated with graded numbers of cells of the various lines.
†$P<0.05$ compared to control mice.

Immunization with the primary mFS6 tumor failed to protect against the M3 and M9 lines. To ascertain whether this was due to loss of antigenicity or to different specificities on these metastatic cell lines, in reciprocal experiments mice were immunized with the nine lines derived from metastases and challenged with either the same cells or the primary mFS6 tumor. Table 6 shows a typical experiment in which M3, M7 and M9 were tested. Under these conditions immunization against M3 and M9 failed to protect mice against both the same lines and the primary

Table 6. Immunogenicity of tumor cell lines derived from lung metastases of the mFS6 sarcoma

Mice immunized against*	Challenge	Takes/total Control mice	Immune mice
M3	M3 10^3 cells	2/2	2/2
	10^4 cells	7/7	7/7
	10^5 cells	5/5	5/5
	mFS6 10^3 cells	2/2	2/2
	10^4 cells	7/7	7/8
	10^5 cells	7/7	6/6
M7	M7 10^4 cells	6/6	6/12†
	mFS6 10^4 cells	6/6	6/15†
M9	M9 10^3 cells	4/4	4/4
	10^4 cells	4/4	4/4
	mFS6 10^3 cells	4/4	4/4
	10^4 cells	4/4	4/4

*Mice were immunized against cell lines from metastases and challenged with either the same metastatic cells or with the primary mFS6 tumor.
†$P<0.05$ compared to control mice.

mFS6 tumor, whereas M7 protected against challenge with M7 and the primary neoplasm. Similar results were obtained in a limited series of experiments in which antigenicity of cell lines from metastases was evaluated by concomitant immunity (data not shown).

DISCUSSION

Results presented show that cell lines from individual lung metastases of a transplanted murine sarcoma are heterogenous with regard to several biological properties. Two (M4 and M7) of nine lines derived from metastases had a greater and two (M8 and M9) had less capacity for disseminating spontaneously to the lungs, compared to the primary mFS6 tumor. Two lines (M2 and M4) gave more artificial lung nodules than the primary sarcoma upon i.v. inoculation, and four lines (M1, M5, M6 and M8) were less efficient than the primary in this respect. Therefore there was little correlation between the metastasizing capacity of the various lines upon i.m. or i.v. inoculation. This lack of correlation may be related to the biological processes necessary for cells at the primary tumor site to reach the blood compartment [12] and/or to the different modifications of haemostasis associated with artificial and spontaneous metastatization [13, 14].

Pooled metastases from the Lewis lung carcinoma reportedly express antigenic specificities different from the primary tumor [11]. On the other hand, Sugarbaker and Cohen [8] reported that tumor lines from individual metastases of a chemically-induced sarcoma were heterogeneous with regard to antigenicity. In the present study two lines (M3 and M9) from metastases had no demonstrable antigenicity and the remaining secondaries had immunological properties similar to the primary tumor, as assessed by *in vivo* transplantation tests. These results, showing that cells from some lung metastases can differ significantly in antigenicity from the remaining secondaries and from the primary tumor, caution against the use of pooled metastases for experimental studies. The lack of antigenicity of a minority [2] of cell lines from metastases has potential implications for therapeutic attempts involving stimulation of specific immunity.

Cells within murine solid tumors differ dramatically in their metastasizing capacity [4, 5], an observation confirmed in the mFS6 model. On this basis, it has been proposed that metastases arise from variant cells preexisting within the primary neoplasm, capable of surviving a strongly selective multistep process [3, 4]. This hypothesis, which predicts that tumor cells from spontaneous metastases would have greater metastatic potential than the primary tumor, was not verified in the present investigation. Hence, the alternative possibility that, at least in some tumors and/or at some anatomical sites, metastasis is not the expression of a strong selection of variant cells still merits serious consideration. Extension of these studies to other murine tumor systems, currently in progress, should provide data relevant to this crucial question.

Acknowledgement—We thank Dr. A. Anaclerio for discussion and criticism.

REFERENCES

1. R. W. Baldwin (Ed.), *Secondary Spread if Cancer.* Academic Press, London (1978).
2. L. Weiss (Ed). *Fundamental Aspects of Metastasis.* Elsevier, Amsterdam (1976).
3. I. J. Fidler, Tumor heterogeneity and the biology of cancer invasion and metastasis. *Cancer Res.* **38,** 2651 (1978).
4. I. J. Fidler and M. L. Kripke, Metastasis results from preexisting variant cells within a malignant tumor. *Science* **197,** 893 (1977).
5. M. L. Kripke, E. Gruys and I. J. Fidler, Metastatic heterogeneity of cells from an ultraviolet light-induced murine fibrosarcoma of recent origin. *Cancer Res.* **38,** 2962 (1978).
6. A. Mantovani, Effects on *in vitro* tumor growth of murine macrophages isolated from sarcoma lines differing in immunogenicity and metastasizing capacity. *Int. J. Cancer* **22,** 741 (1978).
7. F. Spreafico, A. Vecchi, A. Mantovani, A. Poggi, G. Franchi, A. Anaclerio and S. Garattini, Characterization of the immunostimulants levamisole and tetramisole. *Europ. J. Cancer* **11,** 555 (1975).
8. E. V. Sugarbaker and A. M. Cohen, Altered antigenicity in spontaneous pulmonary metastases from an antigenic murine sarcoma. *Surgery* **72,** 155 (1972).

9. L. I. Goldman, B. A. Flaxman, G. Wernick and J. B. Zabriskie, Immune surveillance and tumor dissemination: *In vitro* comparison of the B16 melanoma in primary and metastatic form. *Surgery* **76,** 50 (1974).
10. R. P. Faraci, *In vitro* demonstration of altered antigenicity of metastases from a primary methylcholanthrene-induced sarcoma. *Surgery* **76,** 469 (1974).
11. M. Fogel, E. Gorelik, S. Segal and M. Feldman, Differences in cell surface antigens of tumor metastases and those of the local tumor. *J. nat. Cancer Inst.* **62,** 585 (1979).
12. L. A. Liotta, J. Kleinerman, P. Catanzaro and D. Rynbrandt, Degradation of basement membrane by murine tumor cells. *J. nat. Cancer Inst.* **58,** 1427 (1977).
13. A. Poggi, N. Polentarutti, M. B. Donati, G. de Gaetano and S. Garattini, Blood coagulation changes in mice bearing Lewis lung carcinoma, a metastasizing tumor. *Cancer Res.* **37,** 272 (1977).
14. M. B. Donati, A. Poggi, L. Mussoni, G. de Gaetano and S. Garattini, Hemostasis and experimental cancer dissemination. In *Cancer Invasion and Metastasis: Biologic Mechanisms and Therapy.* (Edited by S. B. Day, W. P. L. Myers, P. Stansly, S. Garattini and M. G. Lewis) p. 151. Raven Press, New York (1977).