Properties of Metastatic and Nonmetastatic Cloned Subpopulations of an Ultraviolet-Light–Induced Murine Fibrosarcoma of Recent Origin

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The present studies were designed to evaluate whether tumor cell properties such as growth rate, chromosome number, anchorage-independent growth, susceptibility to lymphocyte- or macrophage-mediated lysis *in vitro*, and antigenicity *in vivo* correlated with metastatic potential. A murine fibrosarcoma of recent origin induced in a C3H$^-$ mouse by chronic irradiation with ultraviolet light was used. Cells from the parent tumor and its clones were grown in culture. No single property of tumor cells that was measured *in vitro* or *in vivo* predicted or correlated with their metastatic potential. In order for metastasis to occur, all steps of the process must be completed. Therefore, interruption of the sequence at any stage can prevent the production of visible metastasis. It was concluded that the search for a single property common to all metastatic cells in a large variety of neoplasms is likely to be unproductive. (Am J Pathol 97:633–648, 1979)

MOST NEOPLASMS can be conveniently divided into three major categories: a) benign tumors that are noninvasive and nonmetastatic, b) invasive but not metastatic tumors (eg, carcinoma *in situ*, basal cell carcinoma), and c) metastatic neoplasms. Benign tumors are characterized by a structure that is often typical of the tissue of origin; they are thought to be well differentiated and to grow slowly. Mitotic figures are infrequent, and those present are usually normal. In contrast, malignant tumors are usually undifferentiated and consist of a large percentage of dividing cells. These dividing cells may have many abnormal chromosomes, a greater number of chromosomes, and varying degrees of anaplasia.[1]

Little is known regarding the properties of tumor cells that determine their biologic behavior *in vivo*. Metastasis involves the release of cells from the primary tumor, dissemination to distant sites, arrest in the microcirculation of organs, extravasation and infiltration into the stroma of those organs, and survival and growth into new tumor colonies. The outcome of the process has been shown to depend on both host factors and tumor cell properties, and these may vary among tumor systems.[2–4] De-

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spite numerous observations, it is still unclear which properties of tumor cells are associated with or are necessary for the successful development of a metastasis. This question cannot be answered by comparisons drawn among tumors of various histologic types and of possible different etiologies obtained from different strains and even species of animals. Instead, studies must be performed on isolated neoplastic cell lines that have a defined biologic behavior *in vivo*.

Recently we reported that a murine tumor of recent origin is heterogeneous and that cells with widely different metastatic potential pre-exist in the parental populations.[5] We used a fibrosarcoma induced in a C3H/HeN(MTV$^-$) female mouse by chronic ultraviolet (UV) irradiation.[6] The primary tumor was transplanted once into syngeneic mice immunosuppressed by adult thymectomy and sublethal x-irradiation. Four weeks after transplantation, two tumor implants were removed, minced, and trypsinized to prepare a tissue culture line. Cells from the sixth *in vitro* passage of the UV-2237 fibrosarcoma were used to produce clones by a double cloning method. The metastatic behavior of the parent tumor and 21 clones in syngeneic recipients was compared by three different *in vivo* tests. In the first assay, the UV-2237 clones varied greatly in their ability to grow at subcutaneous sites and to produce spontaneous metastases in distant organs. In the second assay, the clones differed in their ability to form pulmonary and extrapulmonary metastases following intravenous injection. The third assay measured the survival time of syngeneic recipients injected intravenously with tumor cells. A strong positive correlation was observed for the ranking of the clones in increasing order of metastatic behavior in the three tests. Clones judged to be nonmetastatic by one test were also considered nonmetastatic by the other two tests.[5]

Because of the availability of a number of cloned subpopulations of the UV-2237 fibrosarcoma which vary in their metastatic behavior *in vivo*, we were able to investigate whether some cellular properties were necessary for metastasis. Specifically, we wished to determine whether the metastatic potential of UV-2237 fibrosarcoma cells was related to their rate of growth *in vitro* and *in vivo*, an abnormal chromosome number, or their resistance to lysis *in vitro* by immune lymphocytes or macrophages. We also investigated the growth and spread of some cloned subpopulations in normal mice and mice treated with UV light.

Materials and Methods

Mice

Specific-pathogen–free mice of the inbred strain C3H/HeN(MTV$^-$) (C3H$^-$) were supplied by the Frederick Cancer Research Center's Animal Production Area.

UV Irradiation

The UV source was a bank of six Westinghouse FS40 sunlamps, which delivered an average dose rate of approximately 2 J/m^2/sec over the wavelength range of 280–340 nm. The mice were housed 5 per cage on a shelf 20 cm below the fluorescent bulbs, and the cage order was systematically rotated before each treatment. The dorsal hair of the animals was removed with electric clippers once a week, and the mice were irradiated for 1 hour, three times per week, until they were used in experiments. The mean time of tumor appearance from this regimen of UV treatment is approximately 32 weeks, with a range of 22 to 42 weeks.[6] None of the animals had developed primary tumors from the UV irradiation at the time of these experiments. Age- and sex-matched untreated mice served as control groups.

Tumor and Tissue Culture Lines

The fibrosarcoma UV-2237 was induced in a female C3H$^-$ mouse by chronic UV irradiation [6] and cloned *in vitro* as detailed elsewhere.[5] Cell cultures were grown in Eagle's minimal essential medium supplemented with 10% fetal calf serum (FCS), vitamin solution, sodium pyruvate, nonessential amino acids, and L-glutamine. No antibiotics were included in the medium used for routine maintenance of the cell lines. The components of this complete medium (CMEM) were obtained from Flow Laboratories (Rockville, Md). All tumor cell lines were tested for and found free of Mycoplasma and the following murine viruses: reovirus type 3, pneumonia virus of mice, K virus, Theiler's encephalitis virus, Sendai virus, minute virus of mice, mouse adenovirus, mouse hepatitis virus, lymphocytic choriomeningitis virus, ectromelia virus, and lactate dehydrogenase virus (Microbiological Associates, Walkersville, Md). In order to ensure the reproducibility of assays, the cultures were grown for 8 weeks and then replaced from frozen stocks. For all the *in vivo* and *in vitro* studies, tumor cells were harvested from subconfluent cultures (50–70% confluent) by rinsing the monolayers with 0.25% trypsin and 0.02% EDTA. After 1 minute, the flasks were tapped to dislodge the monolayers, and the cells were washed in CMEM with 10% FCS. The cells were resuspended in Hanks' balanced salt solution (HBSS) for counting and injection *in vivo*. Only suspensions containing single cells of >90% viability were used for injection.

Experimental Pulmonary Metastasis

Tumor cell suspensions were diluted in HBSS and injected into syngeneic mice via the tail vein in a volume of 0.2 ml. The animals were killed 21 days later; the lungs were removed, rinsed in water, and fixed overnight in Bouin's solution. This procedure permits one to identify the foci of fibrosarcomas, which appear white on a yellow background of uninvolved lung tissue.[7] In mice, most pulmonary metastases are located at the lung surface. Thus, we determined the number of tumor foci by counting the surface metastases under a dissecting microscope.

Ear Assay for Spontaneous Metastasis

Mice under light methoxyflurane (Metofane) anesthesia were given injections in the external ear as described previously.[8] The tumor cells in a 0.1 ml volume were injected into the midportion of the ear (medial surface) with a 27-gauge needle. We left the needle in place for several seconds to allow distribution of the fluid from the injection to prevent egress of the tumor cells. After 1–2 weeks, a small tumor nodule was palpable in the external ear. Tumor incidence and size were measured weekly. Autopsies were performed on moribund or dead animals. Surviving mice were killed, and autopsies were done 8 weeks after subcutaneous tumor cell injection.

In Vitro Growth Rate Determinations

Cell lines were plated at a density of 10^4 cells per 60-mm plastic dish (Falcon Plastics, Oxnard, Calif). Duplicate cultures were trypsinized, and the number of cells per dish was determined once a day for 5 days with the use of a Coulter counter (Coulter Electronics, Inc., Hialeah, Fla).

Growth in Semisolid Medium

A modification of the technique of MacPherson was used.[9] A 4-ml base layer of Autopow CMEM with 10% tryptose phosphate broth containing 0.6% Difco Noble agar was set in 60-mm plastic dishes. This was overlaid with 1.5 ml of a second layer of agar containing 375 or 675 single cells in 0.3% Difco Noble agar. All cultures were in triplicate. Three weeks after plating, the size of all colonies exceeding 0.05 mm in diameter was measured by a microscope equipped with a Filar micrometer eyepiece (American Optical, Buffalo, NY).

Chromosome Analysis

Tumor cells were plated at 10^6 per 100-mm plastic Petri dish, and 24 to 36 hours later the cultures were incubated with 1 μg/ml Colcemid (Calbiochem, La Jolla, Calif), for 2 hours at 37 C. The cells were then harvested and suspended in 0.075 M KCl for 45 minutes. The cells were fixed in methanol–acetic acid (3 : 1) and dropped onto slides. Chromosome counts from at least 100 cells were made on each cell line. The differences in chromosome numbers were analyzed by the Student *t* test, the Mann-Whitney U test, and the Median test.

Immunization of Mice Against UV-2237 Fibrosarcoma

$C3H^-$ mice were given subcutaneous injections of 1×10^6 tumor cells pretreated for 45 minutes with 50 μg/ml mitomycin C and washed three times in HBSS. Two weeks later, the mice were challenged with a lethal dose (1×10^5) of viable UV-2237 cells. Only those mice that rejected the subcutaneous tumor challenge were considered immune and used for the subsequent studies.

Sensitization of Mice to BCG

$C3H^-$ mice were given intraperitoneal injections of 1×10^7 viable BCG organisms (Trudeau Institute, Saranac Lake, NY). Two weeks later, peritoneal exudate cells (PECs) were harvested as described below.

Preparation and Purification of Normal Macrophage Cultures

Pure $C3H^-$ mouse macrophage cultures were prepared as detailed previously.[10,11] Briefly, mice were injected intraperitoneally with 2 ml thioglycollate and killed after 5 days. Their PECs were harvested by washing with Hanks' balanced salt solution (HBSS), $Ca^{++}Mg^{++}$-free. The PECs were centrifuged, resuspended in serum-free MEM, and plated into 16-mm-well plastic dishes (Falcon Plastics, Oxnard, CA) at a concentration of 1×10^6 PECs per well. After forty minutes of incubation at 37 C, the cultures were washed, and all nonadherent cells were removed. Adherent cells were refed with CMEM and incubated for 24 hours. At that time most of the adherent cells (50% of originally plated cells) had the typical macrophage morphology and phagocytosed carbon particles. These cells were used in the *in vitro* cytotoxicity assays.

In Vitro Activation of Normal Mouse Macrophages by Rat Lymphocyte Supernates

Concanavalin A macrophage-activating factor (Con A MAF) was prepared from cultures of normal F344 rat lymphocytes incubated *in vitro* with Con A bound to Sepharose beads (Pharmacia, Piscataway, NJ) as described elsewhere.[12] $C3H^-$ macrophages were in-

cubated with the lymphocyte supernates (Con A MAF) for 24 hours at 37 C. All macrophage cultures were then washed with CMEM prior to the addition of target cells.

Harvest and Preparation of Immune or BCG-Sensitized Macrophages

Immune or BCG-stimulated mice were given intraperitoneal injections of thioglycollate, and PECs were harvested and cultured as described above. Forty minutes after incubation at 37 C, the cultures were washed, and labeled target cells were added to the wells.

Assay for Macrophage-Mediated Cytotoxicity *In Vitro*

Macrophage-mediated cytotoxicity *in vitro* was studied by a radioactive release assay.[10,11] Target cells in exponential growth phase were labeled *in vitro* for 24 hours with 0.2 μCi of ^{125}I-5-iodo-2′-deoxyuridine (^{125}IUdR; 2000 Ci/mmol) per milliliter medium (New England Nuclear Corporation, Boston, Mass). After the labeling period and before the assays, the cultures were washed several times with MEM to remove all unbound radioactive label. Following a 1-minute typsinization, the labeled cells were washed and resuspended in CMEM. Ten thousand labeled target cells were added to each 16-mm culture well containing the previously plated macrophages, yielding an initial ratio of macrophages to target cells of 50 : 1. At this ratio, normal (nonactivated) macrophages are not cytotoxic to neoplastic cells, whereas activated macrophages are cytotoxic.[13] Target cells were also plated alone, as an additional control group. Twenty-four hours after plating, the cultures were washed and refed with CMEM to remove target cells that did not plate. On day 3 or 4 the cultures were washed twice with HBSS to remove all nonadherent cells. The remaining adherent, viable cells were lysed with 2 ml 0.1 N NaOH, and the lysate and washes were monitored in a gamma counter. Maximal *in vitro* macrophage-mediated cytotoxicity in this assay is obtained after 3–4 days of incubation with target cells, and macrophages do not reincorporate the ^{125}IUdR released from dead target cells.[13]

Preparation of Lymphocytes

Axillary, cervical, and mesenteric lymph nodes and spleens were collected from normal or immunized mice in HBSS and forced through an 80-mesh wire sieve (E-C Apparatus, St. Petersburg, Fla). The resulting suspensions were filtered through gauze and passed over a glass wool column to remove adherent cells. Red blood cells were lysed, and the nonadherent lymphoid cells were washed and resuspended in CMEM. Nearly all of these cells were determined morphologically to be lymphocytes, with a viability of 90–95%, as determined by trypan blue exclusion.

Lymphocyte-Mediated Cytolysis

Lymphocytes from normal or immune C3H$^-$ mice were mixed with ^{125}IUdR-labeled target cells. The mixtures containing 1×10^4 target cells and 1×10^7 lymphocytes (effector-to-target ratio of 1000 : 1) per milliliter were rotated for 30 minutes and then plated into 16-mm-well culture plates. The cultures were refed once, 24 hours after plating, and harvested and monitored for radioactivity 3 days later, as described above for the macrophage-mediated cytotoxicity assay.

Calculation of Specific Lymphocyte or Macrophage-Mediated Cytotoxicity

The percentage of specific cytotoxicity was computed by the following formula:

$$\% \text{ specific cytotoxicity} = 100 \times \frac{\text{cpm in target cells with normal effector cells} - \text{cpm in target cells with test effector cells}}{\text{cpm in target cells with normal effector cells}}$$

The percentage of specific cytotoxicity was analyzed for statistical significance by the Student *t* test (two-tailed).

Results

Tumor Growth Rate Versus Metastatic Potential

In spite of the wide variation in the metastatic behavior of the fibrosarcoma clones (Table 1), their doubling times in monolayer cultures did not differ significantly from each other. We measured the doubling times of 7 clones and found them to range from 17 to 24 hours. Since the growth rate of tumor cells in culture need not reflect the rate of growth of the cells in an animal host, we also measured the growth rate of the tumors at a subcutaneous site in syngeneic mice. Again, there was no direct relationship between the average tumor size measured at different weeks after injection and the metastatic behavior of the tumors. The rapid growth of tu-

Table 1—Spontaneous and Experimental Metastasis Produced by Parental UV-2237 Fibrosarcoma and Its Cloned Subpopulations

	Intravenous*		Subcutaneous†		
Cell source (clone)	n	Median (range) number of pulmonary metastases	Incidence of growth	Average tumor size at 2 weeks (cu mm)	Incidence of spontaneous metastasis[2]
Parent line	34	160.5 (17–300)	5/5	382	2/5
C-15	10	1 (0–9)	3/5	16	0/5
C-38	10	2 (0–8)	1/5	0	0/5
C-46	10	2.5 (0–10)	0/5	0	0/5
C-42	10	5 (0–39)	4/5	0	0/5
C-18	10	8.5 (2–55)	5/5	95	0/5
C-43	10	9.5 (0–78)	5/5	465	1/5
C-27	10	10.5 (0–93)	5/5	401	3/5
C-33	10	17 (0–65)	5/5	132	4/5
C-41	10	20 (0–66)	4/5	10	3/5
C-26	10	25.5 (4–212)	5/5	666	3/5
C-5	10	29 (8–105)	5/5	824	3/5
C-22	10	31 (2–60)	5/5	237	2/5
C-9	10	34 (0–104)	5/5	616	1/5
C-47	8	94 (10–297)	5/5	566	2/5
C-34	10	104 (38–179)	5/5	245	4/5
C-44	10	112.5 (64–364)	5/5	389	2/5
C-30	10	126.5 (17–213)	5/5	272	2/5
C-31	10	128.5 (56–185)	5/5	437	3/5
C-39	10	135 (85–248)	5/5	853	2/5
C-25	10	140.5 (79–300)	5/5	132	4/5

* Eighteen days after intravenous injection of 1×10^5 tumor cells. Six-week-old mice killed 21 days after injection. The number of pulmonary metastases was determined with a dissecting microscope.

† Mice were given 1×10^6 cells subcutaneously on the flank. Mice without tumors were observed for 5 months.[5]

mors subcutaneously was not necessarily associated with high metastatic potential. Some highly metastatic clones actually grew more slowly than less metastatic clones.

The ability of cells to grow progressively while suspended in a semisolid medium generally distinguishes transformed (tumorigenic) from normal (nontumorigenic) cells.[14,16] This property of tumorigenic cells as well as their lack of contact inhibition of growth and low serum requirements have been attributed to an increase in autonomy from growth regulatory mechanisms.[14,16] We investigated whether the ability of cells to grow in agar might also correlate in some way with their metastatic potential. Cells of several clones with different metastatic potential were plated into 0.3% Difco Noble agar. Plating efficiencies were determined 21 days later. Between 9 and 14% of plated cells from metastatic and nonmetastatic clones developed into colonies exceeding 0.05 mm in diameter. Thus we were unable to make a distinction between metastatic and nonmetastatic cells based upon their ability to form colonies in a semisolid suspension medium.

Chromosome Number Versus Metastatic Potential

The mode and range of chromosome numbers of several cloned lines are shown in Table 2. With one exception (Clone 38), neither the modes of the different clones (which were very similar) nor the differences in ranges correlated with metastasis. Clone 38 exhibited a different chromosome pattern from the others, ie, hyperploidy, with a wide range in chromosome number. Nonetheless, Clone 38 is virtually nonmetastasizing. Furthermore the ranges in chromosome number from at least 100 cells of clones 25 and 15 (high and low metastatic potential, respectively) are indistinguishable. No individual clone differed significantly from the general population of all other clones (except Clone 38) by the Student *t* test, the Mann-Whitney test, or the Median test.

Table 2—Chromosome Mode and Range of UV-2237 Fibrosarcoma Clones

Clone no.	Chromosome*	
	Mode	Range
15	39	23–68
38	16–66	24–100
42	40	21–71
31	39	27–72
26	41	19–63
12	39	30–69
25	40	19–85

* Average of 100 or more chromosome spreads per clone.

Susceptibility of Clone Lysis Mediated *in Vitro* by Syngeneic Lymphocytes

At an effector-to-target ratio of 1000:1, lymphocytes obtained from normal C3H$^-$ mice were not cytotoxic to any of the UV-2237 cloned lines. In contrast, by 72 hours of co-cultivation an equal number of lymphocytes obtained from mice immunized against the parent UV-2237 tumor lysed the target cells. Specific cytotoxicities ranged from 22% to 41%. At the same time, allogeneic B16 melanoma cells (of C57BL/6 origin) were unharmed (Table 3). This experiment was performed three times, to yield similar results. However, the ranking of the target cell lines according to their relative susceptibility to lymphocyte-mediated lysis *in vitro* varied. We are therefore unable to correlate the metastatic potential of the cloned lines *in vivo* with their resistance or susceptibility to lymphocyte-mediated lysis *in vitro*.

Susceptibility to *in Vitro* Lysis by Macrophages Versus Metastatic Potential

The preceding experiment demonstrated that all clones of the UV-2237 fibrosarcoma were susceptible to *in vitro* lysis by syngeneic lymphocytes obtained from mice immunized against the parental tumor. In parallel experiments we tested whether the parent tumor and its clones were susceptible to the cytotoxic effects of syngeneic macrophages obtained from either tumor-immune or BCG-stimulated mice. The data from a representative experiment are shown in Table 4. Macrophages collected from normal syngeneic (C3H$^-$) mice were not cytotoxic to most of the tested clones with the exception of Clones 39 (11% cytotoxicity) and 34 (15% cytotoxicity). Macrophages obtained from C3H$^-$ mice immunized

Table 3—*In Vitro* Cytolysis Mediated by Lymphocytes From Normal or Immune Syngeneic Mice Against Parental UV-2237 Fibrosarcoma and Several of Its Cloned Subpopulations

Source of tumor target*	Percentage of lymphocyte mediated cytotoxicity by†			
	Normal lymphocytes		Immune lymphocytes	
	48 hr	72 hr	48 hr	72 hr
Parent UV-2237 fibrosarcoma	0	0	60	63
Clone 15	0	0	22	20
Clone 46	0	0	38	77
Clone 42	0	0	36	62
Clone 43	0	0	29	74
Clone 39	0	0	41	57

* Total of 10,000 target cells plated. Cells were prelabeled with ^{125}IUdR. Values are means of triplicate cultures harvested on either Day 2 or Day 3 of cultivation. Cultures were refed once, 24 hours after plating. Percent cytotoxicity versus normal lymphocyte control ($P < 0.01$).

† C3H$^-$ mice immune to a lethal dose of UV-2237 fibrosarcoma cells injected subcutaneously. Lymphocyte to target ratio, 1000:1.

Table 4—*In Vitro* Cytotoxicity Mediated by Peritoneal Macrophages from Normal, Immune, or BCG-Stimulated Syngeneic Mice Against Parental UV-2237 Fibrosarcoma and Several of Its Cloned Subpopulations

Source of tumor target*	Percentage of macrophage-mediated cytotoxicity by†		
	Normal macrophages	UV-2237 immmune macrophages	BCG-stimulated macrophages
UV-2237 parent	7	51	64
Clone 15	0	36	47
Clone 38	0	52	50
Clone 46	0	61	72
Clone 42	0	66	70
Clone 43	0	53	68
Clone 12	0	61	81
Clone 39	11	41	80
Clone 34	15	52	77
Clone 25	0	31	63

* Total of 10,000 target cells plated. Cells were prelabeled with ^{125}IUdR. Values are means of triplicate cultures harvested on Day 3 of cultivation. Cultures were refed once, 24 hours after plating. X

† Percentage of cytotoxicity compared with normal macrophages at corresponding ratio to target cells; all values were statistically significant ($P<0.01$).

against the parent tumor or mice given intraperitoneal injections of viable BCG organisms were highly cytotoxic to all the tested targets. Percent *in vitro* cytotoxicity ranged from 31% to 66% for macrophages obtained from immune mice and from 47% to 81% for macrophages obtained from BCG-stimulated animals. The metastatic potential of the UV-2237 clones, therefore, did not correlate with increased resistance to lysis by syngeneic macrophages *in vitro*.

Experimental and Spontaneous Metastasis in Normal and UV-irradiated Recipients

The original experiments characterizing the metastatic potential of the UV-2237 parental line and its cloned subpopulations were performed in normal C3H$^-$ mice. The data shown in Table 1 identify several clones as being nonmetastatic. One reason for the failure of tumor cells to develop into visible metastases could be a high degree of immunogenicity, which would bring about the elimination of the tumor by host defense mechanisms. Since UV-irradiated mice are unable immunologically to reject UV-induced tumors (see Discussion), we compared the pattern of subcutaneous growth and spontaneous and/or experimental metastasis of the parental tumor and several clones following injection into normal or UV-irradiated recipients. In the first experiment, shown in Table 5, we counted the number of experimental metastases produced by the intravenous injection of tumor cells. Cells of the parental tumor and Clones 46, 34, and 39 ($P < 0.005$) produced a significantly higher number of pulmonary metastases in

Table 5—Pulmonary Metastases in Normal and UV-irradiated C3H Mice Following the Intravenous Injection of 100,000 Viable Cells from Syngeneic UV-2237 Fibrosarcoma Parent Line and Its Clones of Differing Metastatic Potential

Source of tumor cells	Median (range) of pulmonary metastases in*		
	Normal C3H$^-$	UV-irradiated C3H$^-$†	$P <$ ‡
Parent UV-2237	50(25–174)	148(96–217)	0.005
Clone 38	0.5(0–12)	1(0–4)	NS
Clone 46	1(0–5)	39(17–64)	0.001
Clone 15	2(1–10)	4(1–12)	NS
Clone 25	66.5(15–90)	91(60–212)	NS
Clone 34	41.5(20–130)	156(134–205)	0.005
Clone 39	51(16–125)	161(142-192)	0.005
Clone 43	18(9–26)	10(9–49)	NS

* Nine or 10 mice per group. Mice were killed 21 days after intravenous injection. The number of pulmonary metastases was determined with a dissecting microscope.

† Mice were treated with UV radiation for 1 hour, 3 time per week, for 7–9 weeks.

‡ Probability of no difference from unirradiated control group (two-tailed Mann-Whitney U test). NS = not significant.

UV-irradiated mice than that produced in normal mice. Cells from Clones 25, 38, 15, and 43 produced comparable numbers of metastases in both normal and UV-irradiated recipients. Of interest is the contrast found between the metastatic potential of cells of Clone 46 and cells of Clones 15 and 38. All three clones were judged to be nonmetastatic in normal mice. Cells of Clones 15 and 38 were not metastatic in normal or UV-irradiated mice. Apparently their failure to produce metastases is not due to a high degree of immunogenicity. On the other hand, cells from Clone 46 produced a large number of tumor colonies in the lungs of UV-irradiated recipients (median number of lung nodules, 39). Apparently, cells of Clone 46 are metastatic but are also highly immunogenic and therefore are destroyed in normal hosts. In UV-irradiated recipients, which cannot immunologically reject UV-induced tumors, their metastatic potential is apparent.

The subcutaneous growth and spontaneous metastasis of the UV-2237 parental line and Clones 25 and 34 (metastatic in normal mice) and Clones 38 and 46 (not metastatic in normal mice) were studied in normal and UV-irradiated C3H$^-$ mice. The parent tumor and Clones 25 and 34 grew well in both normal and UV-irradiated mice and produced metastases in the majority of the animals. Cells from Clone 46 grew poorly and did not metastasize in normal C3H$^-$ mice. Clone 46 grew in all UV-irradiated mice and produced metastases in 2 out of 5 animals. Clone 38 did not grow at all in normal mice. In UV-irradiated animals large tumors grew in 3 out of 5 mice, but metastasis did not occur.

Discussion

The present studies were designed to evaluate whether tumor cell properties such as growth rate, chromosome number, anchorage independent growth, and susceptibility to *in vitro* mediated lysis by lymphocytes and/or macrophages correlated with metastatic potential. We also studied the metastatic potential of the parental tumor and several cloned subpopulations of the UV-2237 fibrosarcoma in normal and UV-irradiated recipients. The major conclusion to be drawn from our studies is that no single property of tumor cells that we measured *in vivo* or *in vitro* always predicts or correlates with metastatic potential.

A change in chromosome number from diploidy to aneuploidy has been implicated in the progression of some tumors from a benign to a malignant stage.[17,18] No correlation was found between the chromosome number and range of cloned populations of cells and metastatic potential *in vivo*. The one case of hyperploidy observed in cells of UV-2237 was associated with Clone 38, which is nonmetastatic in both normal and UV-irradiated mice. Further, both metastatic and nonmetastatic clones of the UV-2237 fibrosarcoma were primarily diploid. Systematic polyploidy was not associated with metastatic behavior in this tumor system. An increased growth rate could be responsible for the eventual dominance of tumor variants with an increasingly malignant character. No correlation between growth rate *in vitro* and metastatic potential *in vivo* could be demonstrated. However, measuring the doubling time of cells *in vitro* does not take into account the possible involvement of host factors such as immunity that could influence tumor growth. We therefore also measured the rate of growth of several UV-2237 clones in normal and UV-irradiated syngeneic mice (Tables 1 and 6). No direct correlation between growth rate of a cell line *in vivo* and its doubling time *in vitro* was demonstrated. Furthermore, there was no simple relationship between the metastatic behavior of the cell lines and their growth rate in animals. Although some of the nonmetastatic clones grew slowly in animals after subcutaneous injection, the highly metastatic clones did not necessarily grow more rapidly.

Experimental studies on the role of host immunity in cancer metastasis have yielded contradictory results. In many transplantable tumor systems depression of host immunity can increase the incidence of experimental and spontaneous metastasis.[19–22] In other tumor systems, however, depression of immunologic reactivity was shown to decrease or even prevent metastasis[23–25] or to have no influence on the growth of a local or disseminated tumor.[26] In the present studies we found no correlation between metastatic potential of the cloned fibrosarcoma cells and their ability to resist lysis mediated *in vitro* by syngeneic lymphocytes or macrophages.

Table 6—Subcutaneous Growth and Spontaneous Metastasis of UV-2237 Fibrosarcoma Parent Line and Its Clones in Normal and UV-Irradiated Syngeneic C3H$^-$ Mice

Recipients	Tumor source*	Cumulative tumor incidence, days after subcutaneous injection†						Total tumors per group	Incidence of metastasis‡
		7	14	21	28	42	60		
Normal C3H$^-$									
	UV-2237 parent	2/5(0.3)	5/5(0.8)	5/5(1.2)	5/5(1.8)	5/5(2.4)	—	5/5	3/5
	Clone 25	2/5(0.2)	5/5(0.5)	5/5(0.7)	5/5(1.0)	5/5(1.6)	2/2(2.5)	5/5	3/5
	Clone 34	2/5(0.2)	2/5(0.4)	4/5(0.4)	4/5(0.9)	4/5(1.5)	3/3(3.0)	4/5	3/5
	Clone 46	0/5	2/5(0.4)	2/5(0.5)	2/5(0.6)	2/5(1.4)	2/5(3.5)	2/5	0/5
	Clone 38	0/5	0/5	0/5	0/5	0/5	0/5	0/5	0/5
UV-irradiated C3H$^-$									
	UV-2237 parent	2/5(0.2)	5/5(1.5)	5/5(1.9)	5/5(2.8)	—	—	5/5	4/5
	Clone 25	2/5(0.2)	5/5(0.8)	5/5(1.5)	5/5(1.8)	3/3(2.9)	—	5/5	3/5
	Clone 34	3/5(0.2)	5/5(0.6)	5/5(0.8)	5/5(1.3)	5/5(1.9)	—	5/5	4/5
	Clone 46	2/5(0.2)	5/5(0.5)	5/5(0.9)	5/5(1.4)	5/5(2.6)	—	5/5	2/5
	Clone 38	0/5	0/5	0.5	2/5(0.5)	3/5(1.4)	3/5(2.7)	3/5	0/5

* Mice were given subcutaneous injections of 500,000 viable tumor cells.

† Number of mice with tumors per number of mice given injections (surviving mice). Numbers in parentheses, mean tumor size in centimeters.

‡ Number of mice with metastases per number of mice given injections.

The lack of metastasis in normal animals injected subcutaneously with tumor cells could be due to many reasons. For example, tumor cells may remain localized at the injection site and thus not reach distant organs. Alternatively, tumor cells might invade lymphatics or blood vessels, reach distant organs, but be incapable of either survival or proliferation in the new environment and therefore fail to develop into visible metastases. Mice exposed to chronic UV irradiation develop single or multiple highly immunogenic fibrosarcomas or squamous cell carcinomas.[6] Many of these tumors are immunologically rejected upon transplantation to normal syngeneic mice but do grow progressively in immunologically deficient mice[6,27] or in syngeneic mice that have been treated for a short period with UV irradiation.[28] It is interesting to note that the immune responses of UV-irradiated mice to a large number of exogenous antigens are normal,[29] including those to allogenic UV-induced fibrosarcomas or even syngeneic chemically induced tumors.[30] The inability of UV-irradiated mice to reject a challenge of syngeneic UV-induced tumors is due, in part, to the presence of suppressor T lymphocytes in their lymphoid organs.[31,32] We determined whether the failure of metastasis observed with some clones of UV-2237 was due to their high degree of immunogenicity, ie, their rejection by host defense mechanism(s). The failure of cells from Clones 38 and 15 to grow in UV-irradiated mice following intravenous injection is not due to their inability to proliferate. Both these tumors grow progressively when injected subcutaneously into UV-irradiated or immunodeficient mice.[28] The failure of these tumors to grow at a subcutaneous site in normal animals is most likely due to their high degreee of antigenicity, but this factor alone cannot acount for their inability to grow following intravenous injection into UV-irradiated mice. Although intravenous injection of tumor cells bypasses the first steps in the metastatic process (ie, invasion of blood or lymphatic vessels and detachment from the primary tumor mass), many subsequent barriers must be overcome before a lung metastasis can be established.[8,33] Thus, by removing an immunologic barrier to tumor growth by transplanting these tumors into UV-irradiated hosts, only one of many steps in the metastatic process has been bypassed. For Clone 46, this immunologic barrier apparently limits metastasis formation in normal mice, since these tumors metastasize readily in UV-irradiated mice. For Clone 38, however, some additional property of the tumor cells prevents the completing of the metastatic process.

In conclusion, the development of metastases appears to be dependent on an interplay between host factors and intrinsic characteristics of the tumor cells.[3,4,33] To establish metastases, tumor cells must complete all steps involved in the metastatic process (Table 7). Enhanced performance

Table 7—The Requirements for the Formation of a Metastasis

Steps of hematogenous metastasis	Neoplasms								
	Benign*	Invasive, nonmetastatic†							Metastatic‡
1. Progressive growth	+	+	+	+	+	+	+	+	+
2. Vascularization	+	+	+	+	+	+	+	+	+
3. Invasion	−	+	+	+	+	+	+	+	+
4. Detachment	−	−	+	+	+	+	+	+	+
5. Embolization (aggregation)	−	−	−	+	+	+	+	+	+
6. Survival in the circulation	−	−	−	−	+	+	+	+	+
7. Arrest in a capillary bed	−	−	−	−	−	+	+	+	+
8. Extravasation	−	−	−	−	−	−	+	+	+
9. Evasion of host defense mechanism(s)	−	−	−	−	−	−	−	+	+
10. Multiplication (progressive growth)	−	−	−	−	−	−	−	−	+

* Do not invade; remain localized.

† Can fail at any of the steps subsequent to invasion.

‡ The "decathlon champion."

of a cell in one step of the process, however, does not compensate for an inability to complete a subsequent step. Metastatic cells are, therefore, analogous to a decathlon athlete who must perform well in every event to succeed.[34] A brilliant performance in one or two events, accompanied by a failure in others, does not ensure success. The failure by most tumor cells to produce metastases need not be due to a single, common factor. Clearly, in order for metastasis to occur, each step of the process must be completed. Interruption of the sequence at any stage can prevent the production of clinical, visible metastasis. Thus, the search for a property uniform to all metastatic cells may be unproductive. Rather, efforts should be made to develop a broad profile of characteristics likely to be associated with the successful metastatic cell.

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