Metastatic Behavior of an Adriamycin-resistant Murine Tumor

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ABSTRACT

The metastatic behavior of a murine fibrosarcoma (UV-2237M-ADR\textsuperscript{M}) resistant to Adriamycin (ADM) (doxorubicin) was investigated. Subclones isolated from the UV-2237M-ADM\textsuperscript{M} line, which originated from a single colony, generally displayed a similar degree of resistance to ADM (eight out of nine clones did not differ from this UV-2237M-ADM\textsuperscript{M} line). In contrast, they differed significantly in their capacity to form lung colonies after i.v. injection (six of nine clones differed significantly from the UV-2237M-ADM\textsuperscript{M} line, \( p \leq 0.005 \), Mann-Whitney \( U \) test). The UV-2237M-ADM\textsuperscript{M} cell line maintained resistance to ADM even after 17 weeks of growth in syngeneic mice, although a gradual decrease in resistance was observed over this time. Spontaneous metastases from the UV-2237M-ADM\textsuperscript{M} tumor commonly retained resistance to ADM. Of 18 cell lines, each established from an individual lung nodule, 16 showed plating efficiencies in the presence of ADM comparable to that of the primary UV-2237M-ADM\textsuperscript{M} tumor. The remaining two lines had partially reverted to the sensitive state. The i.v. administration of ADM significantly reduced the lung tumor burden of mice with ADM-sensitive UV-2237M tumors but failed to affect the lung tumor burden of mice with UV-2237M-ADM\textsuperscript{M} tumors.

The UV-2237M-ADM\textsuperscript{M} tumor line, exhibiting as it does both drug-resistant and metastatic behavior, provides a useful model system with which to investigate the metastatic process and the development of drug resistance.

INTRODUCTION

Improved surgical and radiological protocols have brought about substantial advances in the treatment of primary cancers. However, as Schabel (30) has indicated, more than 50% of malignant tumors in man have already metastasized by the time these characteristics; most investigations into the responses of tumors to specific drugs have used primary tumors only (31, 33). Recent work demonstrating that not all cells within a tumor population are uniform (3, 11, 12, 18, 19, 27) has suggested that the response of metastatic cells to antineoplastic agents might not be identical to the response of cells of the primary tumor. Indeed, this has been shown to be the case (14, 32, 36, 38). Furthermore, among metastases of the same primary tumor, similar variations with respect to chemosensitivity have also been demonstrated (32, 38).

In a previous report, we described the isolation and preliminary characterization of an ADM\textsuperscript{R}-resistant murine fibrosarcoma (15). In this study, we describe the in vivo behavior of a metastatic, ADM-resistant line of a murine fibrosarcoma selected by identical techniques. This line is shown to be spontaneously and experimentally metastatic, and the recovered lung metastases retain their resistance to ADM.

MATERIALS AND METHODS

Animals. Specific-pathogen-free C3H/HeN mammary tumor virus-negative (C3H/HeN\textsuperscript{N}) mice 8 to 10 weeks old were obtained from the Animal Production Area of the NCI-Frederick Cancer Research Facility, Frederick, Md. Within each single experiment, mice were age and sex matched.

Tumor Lines. The parent line UV-2237M, syngeneic to C3H/HeN mice, is a highly metastatic variant isolated from the UV-2237 fibrosarcoma (23) by in vivo selection techniques (12). The ADM-resistant line UV-2237M-ADM\textsuperscript{M} was selected and isolated in vitro by the exposure of the parent UV-2237M line to the continuous presence of ADM as described previously (15). It was originated from a single colony and expanded to bulk culture in increasing concentrations of ADM.

Culture Conditions. The UV-2237M line was maintained on tissue culture plastic in CMEM (Flow Laboratories, Inc., Rockville, Md.). The UV-2237M-ADM\textsuperscript{M} line was cultured in CMEM containing 1 \( \mu \)g of ADM per ml. Both tumor lines were subcultured weekly; the cells were harvested by a brief exposure to 0.25% trypsin-0.02% EDTA. Cell lines were examined routinely for and found to be free of Mycoplasma, reovirus type 3, pneumonia virus of mice, K-virus, Thielier's virus, Sendai virus, minute virus of mice, mouse adenovirus, mouse hepatitis virus, lymphocytic choriomeningitis virus, ectromelia virus, and lactate dehydrogenase virus (Microbiological Associates, Bethesda, Md.). Cell lines were grown from frozen stocks and, except as noted in "Results," were maintained in continuous culture for periods not exceeding 60 days.

Drugs. The anthracycline antibiotic ADM was a gift of Dr. J. Douros, Division of Cancer Treatment, National Cancer Institute, NIH, Bethesda, Md. Stock solutions in 0.9% NaCl (1 mg/ml) were prepared at \(-20^\circ\) and diluted in CMEM to the required concentration immediately prior to use. Stock solutions were not stored for longer than 4 weeks.

For the in vivo administration of ADM, the drug was freshly dissolved in 0.9% NaCl solution at a concentration sufficient to allow inoculum volumes of 0.1 ml/10 g body weight.

Cloning Procedures. To isolate clones, UV-2237M-ADM\textsuperscript{M} cells were harvested with trypsin-EDTA solution and adjusted to a concentration of 5 viable cells/ml of CMEM. A 0.1-ml portion of the cell suspension was seeded into 60-mm petri dishes. Plates were then incubated for 10 days. Surviving colonies were then picked with a sterile Pasteur pipette into 1 ml of CMEM supplemented with 10\% fetal bovine serum and 0.5 mg/ml of L-glutamine.

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plated into each well of 96-well Microtest III tissue culture plates (Falcon Plastics, Oxnard, Calif.), and each well was examined with the aid of an inverted microscope. Those wells containing a single cell were identified; when clones had developed from these cells, they were harvested and expanded in tissue culture vessels of increasing size. Uncloned populations of the UV-2237M-ADM<sup>9</sup> line were maintained in tissue culture for an equal length of time to serve as control populations for determination of the plating behavior in the presence of ADM.

**Determination of ADM Resistance by Colony Formation.** Curves of the dose-response of the different clones and tumor cell lines to ADM were established as described previously (15). Briefly, 800 viable tumor cells were plated into 60-mm tissue culture dishes (Falcon Plastics) containing 5 ml of CMEM with varying concentrations of ADM (triplicate dishes/dose). The cultures were incubated in a humidified atmosphere with 5% CO<sub>2</sub> at 37°C for 8 to 10 days. Culture fluid was discarded; then tumor colonies were fixed and stained with methylene blue in 50% methanol and counted with the aid of a dissecting microscope. Relative plating efficiencies were determined as:

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\frac{\text{Mean no. of colonies in treated dishes}}{\text{Mean no. of colonies in control dishes}} \times 100
\]

**Experimental Metastasis Formation.** Syngeneic C3H/HeN<sup>+</sup> mice were inoculated i.v. via the lateral tail vein with 1 x 10<sup>6</sup> viable (as determined by trypan blue exclusion) tumor cells in single-cell suspension in 0.2 ml of Hanks’ balanced salt solution. Mice were killed 3 weeks after tumor cell injection, lungs were removed and fixed in Bouin’s solution, and the number of peripheral lung tumor nodules was determined with the use of a dissecting microscope. UV-2237M-ADM<sup>9</sup> cells were grown for 7 to 10 days in drug-free medium prior to their injection into animals.

The response of experimental lung metastases to ADM was evaluated by injecting mice i.v. with ADM at a concentration of 10 mg/kg 3 days after the injection of tumor cells. Mice in the control groups received i.v. injections of equal volumes of 0.9% NaCl solution.

**Isolation and Recovery of Tumor Cells from Primary Tumors or Spontaneous Metastases.** Syngeneic C3H/HeN<sup>+</sup> mice were given s.c. injections in the flank region of 2 x 10<sup>5</sup> UV-2237M parent or UV-2237M-ADM<sup>9</sup> tumor cells. At the times designated in “Results,” the mice were killed (5 mice/group), s.c. tumors were excised and dissected free of nonmalignant fat and connective tissue, and the tumors were minced into 1-cm<sup>3</sup> portions using sterile scalpel blades. This tumor tissue was dissociated by 2 sequential 15-mm exposures to 0.25% collagenase-0.01% DNase (Sigma Chemical Co., St. Louis, Mo.), and the obtained single-cell suspensions were plated into 100-mm tissue culture dishes in CMEM and grown as monolayer cultures. Preliminary experiments showed that this technique produced plating efficiencies in the presence of ADM comparable to those obtained with cells obtained with mechanical dissection alone.

Ten to 14 days after being established in tissue culture, the cells obtained from either the primary tumors or the secondary lung tumors were tested for their resistance to ADM by use of the colony-forming assay as described.

In all experiments, UV-2237M-ADM<sup>9</sup> and UV-2237M parent cells that had been maintained in tissue culture were also tested to serve as in vivo controls.

**Statistical Analyses.** Differences in the number of lung colonies were analyzed using the Mann-Whitney U test. Inspection of the individual dose-response curves of the UV-2237M-ADM<sup>9</sup> metastases indicated that 2 of the metastases (Metastases 6 and 30) were much more resistant to ADM than the others. Individual curves of the dose-response to ADM were analyzed to determine whether Metastases 6 and 30 qualified as statistical “outliers” (10). The low probability values obtained (p < 0.01) strongly suggest that these 2 populations do represent outliers, and their behavior is presented separately (Chart 4).

**RESULTS**

**In Vivo Retention of Resistance to ADM.** The retention of the ADM resistance phenotype after prolonged in vitro or in vivo cultivation is demonstrated in Chart 1. Compared with the UV-2237M parent line, the UV-2237M-ADM<sup>9</sup> line showed approximately a 100-fold increase in resistance to the cytotoxic effects of ADM (Chart 1A). In vitro cultivation of the UV-2237M-ADM<sup>9</sup> cells in the absence of ADM led to a diminution in the degree of resistance, but, even after 17 weeks of continuous culture in nonselective CMEM, the UV-2237M-ADM<sup>9</sup> line was still markedly more resistant to ADM than the parent line (Chart 1A). Similarly, cells from the UV-2237M-ADM<sup>9</sup> line that were recovered from s.c. tumors 4, 10, and 17 weeks after injection into syngeneic mice showed a reduction in their resistant behavior compared to the original inoculum, but at 17 weeks, they still retained a marked (10-fold) resistance as compared to UV-2237M parent cells maintained under the same conditions (Chart 1B). These levels of resistance were obtained by comparing values at 10% relative plating efficiencies. Behavior of the UV-2237M parent cells with regard to plating efficiency in medium containing ADM was the same at all tested times after s.c. injection and after extended time in tissue culture (data not shown).

**ADM Resistance and Metastatic Behavior of Clones Isolated from UV-2237M-ADM<sup>9</sup>.** The originally isolated UV-2237M-ADM<sup>9</sup> line was established from a single colony, but the extended culture time required to expand the population could have allowed the introduction of phenotypic diversity. Numerous clones that were derived from the parent UV-2237M-ADM<sup>9</sup> population approximately 4 months after the initial establishment of the line were examined for their in vitro resistance to ADM and for their lung-colonizing capacity. Of 9 clones tested, 8 were indistinguishable from the UV-2237M-ADM<sup>9</sup> line with regard to ADM resistance (Chart 2). The remaining clone (clone 1) was significantly different (p < 0.01 as determined by outlier analysis) (10) from the parent line and the other 8 clones but still, in spite of a decrease in resistance, maintained a more resistant behavior to ADM than the UV-2237M cells (Chart 2).

**Statistical Analyses.** Differences in the number of lung colonies were analyzed using the Mann-Whitney U test. Inspection of the individual dose-response curves of the UV-2237M-ADM<sup>9</sup> metastases indicated that 2 of the metastases (Metastases 6 and 30) were much more resistant to ADM than the others. Individual curves of the dose-response to ADM were analyzed to determine whether Metastases 6 and 30 qualified as statistical “outliers” (10). The low probability values obtained (p < 0.01) strongly suggest that these 2 populations do represent outliers, and their behavior is presented separately (Chart 4).
In contrast to this relatively homogeneous behavior with regard to ADM resistance, the clones displayed heterogeneous behavior with regard to their lung-colonizing capacity. Results from one of 3 similar experiments are presented in Table 1. The injection of 10^5 cells of the UV-2237M-ADM line produced a median of 22 pulmonary tumors 3 weeks later (range, 10 to 58); 6 of the 9 clones displayed a metastatic capacity that was significantly different from the UV-2237M-ADM line cell line (3 at p < 0.005 level and 3 at the p < 0.001 level). There appeared to be no correlation between the ability to form experimental metastases and resistance to ADM (Table 1; Chart 2).

**Effect of ADM Treatment on Lung Tumor Burden.** Mice were inoculated i.v. with 10^5 viable cells of the UV-2237M or UV-2237M-ADM lines, and 3 days later the animals were given a single i.v. injection of ADM (10 mg/kg). Control mice received equal volumes of 0.9% NaCl solution. The combined results of 2 separate but comparable experiments are presented in Table 2. A single dose of ADM significantly reduced the median number of lung colonies produced by UV-2237M cells from 41 (range, 0 to 165) to 6 (range, 0 to 52). In contrast, the same dose of ADM failed to have any effect on the lung-colonizing capacity of UV-2237M-ADM cells (median of 29 pulmonary nodules in treated animals versus 21 in controls; Table 2).

**Retention of ADM Resistance in Spontaneous Metastases.** Eighteen individual cell lines were established from independent lung nodules that arose as a result of spontaneous metastasis of s.c. UV-2237M-ADM; 9 cell lines were derived from individual spontaneous metastases of UV-2237M parent tumors. The plating behavior and resistance profile of these cell lines are shown in Chart 3. Sixteen of the 18 lines exhibited the same resistant behavior as the primary UV-2237M-ADM cell line (Chart 4). Two of the metastases had reverted to the sensitive state (p < 0.01) and thus were indistinguishable from the 9 lines derived from metastases of the sensitive UV-2237M parent tumor (Chart 4).

**DISCUSSION**

The results presented here show that we have been able to isolate and maintain in vitro an ADM-resistant fibrosarcoma cell line that is capable of metastasizing in syngeneic mice. Both experimental (from i.v. inoculation of tumor cells) and spontaneous (from tumor cells implanted s.c.) metastases generally retained the ADM resistance phenotype of the UV-2237M-ADM line. This was indicated by the response of lung tumor burden to i.v. administration of ADM (Table 2) and the plating behavior of cells recovered from sites of secondary development (Chart 3). Additional experiments have shown that single doses of ADM of 10 mg/kg or above have a significant inhibitory effect on tumor takes and growth rates of the UV-2237M parent line whereas these same drug doses had no effect on the same parameters of the UV-2237M-ADM line (data not shown). Injection of UV-2237-ADM cells i.v. produced fewer lung nodules than did injection of equal numbers of UV-2237M cells. These differences might reflect variations in growth rates, a factor that might also modulate sensitivity to ADM activity. However, the growth rates...
of the 2 tumor lines under nonselective conditions are indistinguishable (data not shown), and the metastatic behavior of the cell lines appears to be unrelated to growth rates. In addition, there is no correlation between the lung-colonizing capacity and the degree of ADM resistance of the various clones (Table 1; Chart 2). Thus, in this system, the response to ADM is not dependent on growth rates, which makes the UV-2237M-ADM<sup>6</sup> line a most useful model system for the analysis of drug resistance in a metastatic tumor.

Cell lines were established from individual lung metastases 10 weeks after the initial injection of the monoclonally derived UV-2237M-ADM<sup>6</sup> cells into the s.c. site. In spite of this relatively long growth period in the absence of ADM, 16 of 18 metastases retained their resistance to ADM and their plating behavior was indistinguishable from the primary UV-2237M-ADM<sup>6</sup> tumor line (Chart 3). A wide variety of human (3, 32, 37, 41) and experimental (1, 16, 20, 31, 38) neoplasms have been shown to contain subpopulations of cells with different drug sensitivities. Furthermore, variations in drug response between cells that populate metastases and those isolated from the localized primary tumor have been reported (14, 32, 36, 38). This variability in drug response has been shown to extend to differences between individual metastases in experimental murine tumors (38). The original UV-2237M-ADM<sup>6</sup> line was of clonal origin, and it therefore represents a relatively uniform starting population. Although metastases from this line generally exhibit resistance to ADM, some differences in this response do occur, as manifested by the plating efficiencies of Metastases 6 and 30 (Chart 3).

The loss of the ADM resistance phenotype has been reported in some (21, 39) but not all experimental tumors (2, 4, 9). Baskin et al. (2) have equated instability of drug resistance with the presence of double-minute chromosomes; in many of their cell

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Chart 3. Plating behavior of cell lines established from individual lung metastases. a, cell lines derived from metastases of the UV-2237M-ADM<sup>6</sup> line; b, cell lines derived from metastases of the UV-2237M parent line. * indicates primary tumors isolated from mice bearing lung nodules. Points, mean values derived from 2 separate experiments.

Chart 4. Plating behavior of spontaneous metastases in presence of ADM. Geometric means of 16 ADM-resistant (•) and 2 revertant (Metastases 6 and 30) (△) metastatic cell lines obtained from UV-2237M-ADM<sup>6</sup> line. 0, means of 9 metastatic cell lines obtained from UV-2237M parent. The value 0.01 was substituted for 0 to permit the inclusion in the calculations of those metastases with 0% survival at the higher ADM concentrations.

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lines, stability was established in association with loss of these double minutes. However, these structures did not appear to play a role in ADM resistance, which was stable (2). Although our lines were not uniformly stable, we have been unable to demonstrate double-minute chromosomes in the UV-2237M-ADM cell line. We are continuing to examine cells from the various clonal lines for this feature. Such chromosomal markers are known to occur in some drug-resistant human tumors (8). Conceivably, their presence or absence in metastases derived from different clones could be responsible for differences in chemosensitivity exhibited by these metastases.

Many metastases appear to be of clonal origin (25, 35); however, within a short time-span they evolve to exhibit a marked degree of intrasional heterogeneity (28). The rate of formation of metastatic variants has been investigated by Poste et al. (26, 28), who found that this rate was higher in populations containing a limited number of tumor cell populations than in highly heterogeneous, polyclonal populations. Poste et al. (28) noted that, even under conditions which led to the rapid generation of metastatic variants, drug resistance phenotypes remained stable. Thus, cell populations appear to develop heterogeneity for the metastatic phenotype more rapidly than for other phenotypes. Certainly, the findings reported here may well be consistent with this view of the generation of heterogeneity for metastatic ability.

As can be seen in Table 1, 6 out of 9 clones derived from the UV-2237M-ADM line exhibited metastatic behavior that, as assessed by the formation of lung nodules after i.v. injection of tumor cells, was highly significantly different from the behavior of the parent line. As has been demonstrated for a number of other tumor systems (6, 12, 23, 27, 29, 34), the UV-2237M-ADM line is heterogeneous with regard to the metastatic phenotype, and this diversity develops within a few weeks after isolation of the cell line. Looking at the rate and frequency of this generation of metastatic diversity, Harris et al. (17) proposed what they termed the “dynamic heterogeneity” model of tumor metastasis. Noting that metastatic variants in a tumor population arise at a higher rate than drug-resistant variants, these workers suggested that such variants might be generated by epigenetic mechanisms or by specific genetic mechanisms that operate at high rates. Our results, which revealed marked metastatic heterogeneity between the various clones, may be compatible with this hypothesis and with the data of others (17, 28) that indicate that drug resistance phenotypes are relatively more stable than the metastatic phenotype.

Cell lines resistant to ADM have been isolated by numerous workers (4, 7, 9, 15, 21, 22, 24, 39, 40). However, almost none of these drug-resistant tumor lines are also spontaneously metastatic in the appropriate recipient animal. A recent report has described the isolation of ADM-resistant lines from a metastatic murine melanoma (40), but no information was provided regarding the metastatic behavior of the resistant variants. Conversely, while the metastatic behavior of the drug-resistant melanoma variants obtained by Poste et al. (25–28) has been fully documented, there is no information on the degree or mechanisms of this drug resistance. Thus, the UV-2237M-ADM tumor, exhibiting as it does the dual characteristics of spontaneous metastatic capacity and retention of resistance by the resultant secondary tumors, provides a unique model system with which to study these 2 features of neoplastic disease that have such profound relevance to clinical oncology.

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