Isolation and Preliminary Characterization of an Adriamycin-resistant Murine Fibrosarcoma Cell Line

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ABSTRACT

A variant cell line (UV-2237-ADM<sup>R</sup>) resistant to the anthracycline antibiotic Adriamycin (doxorubicin) was selected in vitro from the murine UV-2237 fibrosarcoma tumor cell line. Resistance to Adriamycin proved to be a stable characteristic of the UV-2237-ADM<sup>R</sup> line, whether the line was grown in vivo or in vitro. The UV-2237-ADM<sup>R</sup> line also exhibited increased resistance to N-trifluoracetadriamycin-14-valerate, daunorubicin, actinomycin D, amsacrine, mitomycin C, vinblastine, and vincristine but not to bleomycin. Cell-cell hybridization studies showed that the Adriamycin resistance is an incompletely dominant trait. Uptake and efflux studies with [14C]Adriamycin indicated that the resistance exhibited by the UV-2237-ADM<sup>R</sup> line was due to both reduced uptake of the drug and an increased active efflux.

INTRODUCTION

One of the most significant problems in cancer chemotherapy is the rapid emergence of variant subpopulations of tumor cells that are resistant to a particular drug or combination of drugs. The anthracycline antibiotic ADM<sup>R</sup> (doxorubicin) is commonly used because it exhibits considerable cytotoxic activity against a broad spectrum of solid tumors and leukemias (4-6, 11). Patients frequently develop tumor cells that are resistant to ADM, but the cause of this resistance is unknown.

To study the mechanisms of drug resistance, many investigators have selected in vivo or in vitro mammalian cell lines that are resistant to levels of ADM that are normally cytotoxic (3, 9, 17, 22, 28, 37, 38). Most of these tumor lines have been either leukemias or sarcomas grown in the ascites form; there is very little information in the literature regarding the selection of variant lines from solid tumors. As part of our work on developing experimental therapies of transplantable tumors, we needed to obtain a solid murine tumor that is resistant to ADM. In this initial report, we describe the isolation and partial characterization of a variant of the UV-2237 fibrosarcoma (24) that exhibits such behavior.

MATERIALS AND METHODS

Tumor Lines. The UV-2237 fibrosarcoma, syngeneic to C3H/HeN mammary tumor virus-negative mice, was a gift of Dr. Margaret L. Kripke, National Cancer Institute-Frederick Cancer Research Facility (24). The parent line was maintained in tissue culture in CMEM (Flow Laboratories, Inc., McLean, Va.).

The so-called ‘universal fuser line’ bearing a dominant marker for resistance to ouabain and a recessive marker for resistance to 6-thioguanine was selected from UV-2237 after mutagenesis with ethylmethanesulfonate. This line, UV-2237<sup>R</sup>, was maintained in CMEM containing 3 mM ouabain and 6-thioguanine (10 μg/ml) and was the gift of Dr. Maria Cifone, NCI-Frederick Cancer Research Facility.

Drugs. The antitumor agents ACT D, ADM, AMSA, BLEO, DNR, VBL, and VCR were provided by Dr. J. Douros, National Products Branch, Division of Cancer Treatment, National Cancer Institute, Bethesda, Md. AD 32 was a gift from Dr. M. Israel, Sidney Farber Cancer Institute, Boston, Mass. MIT C was obtained from Bristol Laboratories, Syracuse, N. Y.

BLEO, DNR, MIT C, and VCR were dissolved in distilled water; AMSA was dissolved in 1 mol/lactic acid. ACT D in 95% ethanol, AD 32 in 0.9% NaCl solution containing 10% Tween 80, and ADM in 0.9% NaCl solution. All drugs were dissolved immediately before use, except ADM, which was stored at -20°C in stock solutions of 1 mg/ml for periods of up to 2 weeks. Working concentrations were prepared in CMEM; control medium contained equal volumes of drug-free diluent.

[14C]ADM (92 μCi/mg) was a gift from Dr. Federico Arcamone, Farmitalia Carlo Erba, Milan, Italy.

Culture Conditions. All tumor cell lines were maintained on plastic and subcultured weekly by harvesting cells with 0.25% trypsin-0.02% EDTA. CMEM was modified according to the cell phenotype as described in “Results.” The cell lines were routinely examined for and found 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, lymphocyte choriomeningitis virus, ectromelia virus, and lactate dehydrogenase virus (M. A. Bioproducts, Walkersville, Md.).

Determination of Cell Survival by Colony Formation. Dose-response curves of the different tumor lines to the various agents were established by plating 800 tumor cells into 60-mm tissue culture dishes (Falcon Plastics, Oxnard, Calif.; triplicate dishes/drug dose) containing 5 ml of CMEM with varying drug concentrations. Cultures were incubated at 37°C for 8 days at which time the medium was discarded, and the colonies were fixed, stained with methylene blue in 50% methanol, and counted. Relative plating efficiencies were calculated as

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

\[
\text{Mean no. of colonies in control dishes}
\]

The UV-2237-ADM<sup>R</sup> line was always subcultured once and grown to partial confluency in ADM-free CMEM before being tested.

Cell-to-Cell Hybridization. Cells were hybridized as described by Davidson and Gerald (10). Briefly, 1 x 10<sup>6</sup> UV-2237-ADM<sup>R</sup> cells and 1 x 10<sup>6</sup> UV-2237 cells were plated into a single 60-mm Petri dish containing 4 ml of CMEM, and the cells were cocultivated at 37°C. After 24 hr of incubation, the culture medium was aspirated off, and the cells were

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3 The abbreviations used are: ADM, Adriamycin (NSC 1213127); CMEM, Eagle’s minimal essential medium supplemented with 10% fetal bovine serum, sodium pyruvate, nonessential amino acids, and L-glutamine; ACT D, actinomycin D (NSC 3053); AMSA, amsacrine (NSC 249992); BLEO, bleomycin (NSC 125686); DNR, daunorubicin (NSC 82151); VBL, vinblastine (NSC 49842); VCR, vincristine (NSC 67574); AD 32, N-trifluoroacetadriamycin-14-valerate (NSC 246131); MIT C, mitomycin C (NSC 249880); HAT medium, hypoxanthine (0.1 μM)-aminopterin (4 x 10<sup>-8</sup> M)-thymidine (3.6 x 10<sup>-8</sup> M)-glycine (3 x 10<sup>-8</sup> M).

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ADM-resistant Solid Tumor Line

Treated with 2 ml of 50% (w/v) polyethylene glycol (M, 1000) in serum-free medium for 1 min at room temperature to induce fusion. The polyethylene glycol solution was then removed, and the cells were washed 4 times with Hanks' balanced salt solution, refed with CMEM, and incubated for a further 24 hr. The cells were then harvested with trypsin-EDTA, resuspended in serum-free medium, and plated (4 x 10⁴ cells/plate) into 100-mm tissue culture dishes (Falcon) containing CMEM plus 3% ouabain plus HAT medium. Two to 3 weeks after initiation, growing colonies were isolated with a cloning ring, grown in the selection medium, and karyotyped to confirm their hybrid nature. Three independent isolates were obtained to test the nature of ADM resistance: their karyotypes are presented in Table 1. Control hybrid lines were obtained by fusing UV-2237 and the unselected UV-2237-parent line. Control plating experiments with both UV-2237 and UV-2237-ADM lines that had been cultured in separate dishes demonstrated that no growth occurred in dishes containing 3% ouabain plus HAT medium, even when 2.5 x 10⁶ cells were plated per dish.

In Vitro Growth Curve. Tumor cells from the different lines were seeded into 60-mm dishes (5 x 10⁴ cells/dish) containing 5 ml of the appropriate medium, as described in "Results." Every 24 hr, cells from triplicate cultures were harvested with 0.25% trypsin-0.02% EDTA, and the number of viable trypan blue-excluding cells was determined using a hemocytometer.

In Vivo Growth of the UV-2237-ADM Line. We determined that the ADM-resistant phenotype was maintained after in vivo growth by examining tumor cells that had been recovered after 42 days of growth in the subcutis of nude mice of BALB/c origin. Five mice were given s.c. injections of 1 x 10⁶ UV-2237-ADM cells, and 5 mice were given s.c. injections of 1 x 10⁵ UV-2237 parent cells. Six weeks later, all of the mice were killed; tumors were excised, and single-cell suspensions were prepared (2 sequential 15-min exposures to 0.25% collagenase and 0.01% DNase). These suspensions were plated into 100-mm tissue culture dishes in CMEM and grown as monolayer cultures for 1 week. Seven days later, the cells were harvested and tested in the colony formation assay as described.

Uptake and Efflux Studies. UV-2237 and UV-2237-ADM lines were harvested as described and adjusted in CMEM to give 2.5 x 10⁶ viable cells/ml. Two hundred-μl aliquots of these cell suspensions were added to the wells of 96-well Microtest III tissue culture plates (Falcon), and the plates were washed once with phosphate-buffered saline, 100-μl aliquots of these cell suspensions were added to tubes (Falcon; Model 2058) containing appropriate medium, as described in "Results." Every 24 hr, cells from triplicate cultures were harvested with 0.25% trypsin-0.02% EDTA, and the tubes were inverted and allowed to drain for 2 to 3 hr, after which the cell pellets were lysed with 100 μl of 0.2% sodium dodecyl sulfate and treated as described above. Essentially, the results obtained with these assays were the same as those obtained with the monolayer cultures.

RESULTS

Isolation of the ADM-resistant Line. Early experiments showed that a concentration of 0.1 μg ADM per ml was required to inhibit completely the formation of colonies of the UV-2237 parent line (Chart 1). The resistant variant line was initially selected by plating 5 x 10⁵ viable UV-2237 parent cells (viability

<table>
<thead>
<tr>
<th>Cell line</th>
<th>Chromosome no.</th>
</tr>
</thead>
<tbody>
<tr>
<td>UV-2237 parent</td>
<td>42 ± 3</td>
</tr>
<tr>
<td>UV-2237</td>
<td>37 ± 3</td>
</tr>
<tr>
<td>UV-2237-ADM</td>
<td>36 ± 3</td>
</tr>
<tr>
<td>UV-2237NR x UV-2237 parent</td>
<td>73 ± 4</td>
</tr>
<tr>
<td>UV-2237RR x UV-2237-ADM C13</td>
<td>63 ± 4</td>
</tr>
<tr>
<td>UV-2237RR x UV-2237-ADM C14</td>
<td>66 ± 5</td>
</tr>
<tr>
<td>UV-2237RR x UV-2237-ADM C15</td>
<td>75 ± 5</td>
</tr>
</tbody>
</table>

* Chromosome counts were obtained from at least 30 individual metaphase spreads.

** Mean ± S.D.

Chart 1. Survival of the UV-2237 parent and UV-2237-ADM cell lines plated in various concentrations of ADM. ●, the UV-2237-ADM line selected in the presence of ADM (0.1 μg/ml); ○, the UV-2237 parent at the time of selection; △, the UV-2237 parent maintained in tissue culture for a period required to obtain resistant variants; and ▲, the UV-2237-ADM line maintained in ADM (1.0 μg/ml). -- -- --, no colonies observed at higher concentrations of the drug.
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determined by trypan blue exclusion) per dish into a total of fifty 100-mm tissue culture dishes, each containing 25 ml of CMEM per 0.1 µg of ADM per ml. After 3 weeks of continuous cultivation in this medium, 8 individual colonies developed. These colonies were isolated with the aid of a cloning ring and were grown in fresh medium containing 0.1 µg ADM per ml to mass culture before being frozen. A representative plating experiment with one of these clones is illustrated in Chart 1; it showed that the cell line derived from this first selection was approximately 10 times more resistant to the effects of ADM than was the UV-2237 parent line.

One of these clones was replated into CMEM containing 0.1 µg ADM per ml in a 100-mm dish and then was plated in successive passages into 0.2, 0.5, and 1.0 µg of ADM per ml of medium. This cell line was labeled UV-2237-ADM* and was maintained in CMEM containing 1.0 µg ADM per ml; Chart 1 shows that the UV-2237-ADM* cell line was approximately 100 times more resistant to ADM than was the parent cell line. In the presence of 1.0 µg ADM per ml, the UV-2237-ADM* line was morphologically indistinguishable from the UV-2237 parent line maintained in CMEM containing no ADM (Fig. 1).

The growth-inhibiting effects of ADM and the resistance of the UV-2237-ADM* line to such effects were confirmed by in vitro growth rate experiments (Chart 2). When grown in CMEM, the UV-2237 parent line had a doubling time of approximately 18 hr; when this cell line was cultivated for 7 days in the presence of ADM (0.1 or 1.0 µg/ml), the cells stopped growing and eventually died. In marked contrast, the UV-2237-ADM* line, which had a doubling time of 19 hr when grown in CMEM, was totally unaffected by the incorporation of 0.1 µg ADM per ml into the growth medium; incorporation of 1.0 µg ADM per ml into the culture medium increased the doubling time to 29 hr.

Stability of Resistance. The stable nature of ADM resistance both in vitro and in vivo is shown in Chart 3. The UV-2237-ADM* line maintained in drug-free medium for 6 weeks (6 passages) was less resistant to ADM than was the UV-2237-ADM* line maintained in 1.0 µg ADM per ml (Chart 3A). However, further continuous cultivation of the cell line in drug-free medium for periods of more than 5 months did not lead to any further decrease in these levels of resistance (data not shown). In vivo cultivation of the UV-2237 parent and UV-2237-ADM* tumor lines in the subcutis of nude mice for 6 weeks showed that the recovered UV-2237-ADM* line maintained a 10-fold increase in resistance to the effects of ADM as compared to the recovered UV-2237 parent line (Chart 3B).

Cross-Resistance of the UV-2237-ADM* Cell Line to Other Agents. We tested the resistance of the UV-2237-ADM* line to several agents with different mechanisms of action. Representative results are presented in Chart 4. There was cross-resistance to the closely related analogues AD 32 and DNR; there was cross-resistance also to ACT D, AMSA, MIT C, and the Vinca alkaloids VBL and VCR. No cross-reactivity to BLEO was detected in these assays (Chart 4).

Cell to Cell Hybridization Studies. To test for the dominant or recessive nature of the ADM-resistant trait, cells of the UV-2237-ADM* line were hybridized with ADM-sensitive cells of the UV-2237 parent line, which bears the dominant marker for ouabain resistance and the recessive marker for the absence of xanthine phosphorylase. The hybrids were re-
covered in CMEM containing 3 mM ouabain and HAT medium. The number of metaphase chromosomes of the parent lines was between 36 and 42, whereas that of the hybrids was 63 to 75 (Table 1). The dose-response curves of the ADM-sensitive UV-2237RR line, the UV-2237-ADM R line maintained in drug-free culture for 5 weeks, and 3 independently derived clones resulting from hybridization between these 2 cell types are presented in Chart 5. Also shown in this chart is the dose response of a hybrid resulting from the fusion of cells from the ADM-sensitive UV-2237RR tumor and the UV-2237 parent tumor.

The UV-2237-ADM R line was maintained in drug-free medium for 5 weeks in order to serve as the appropriate control for the hybrid clones that were isolated and grown in medium that did not contain ADM. Chart 5 shows that the UV-2237-ADM R line exhibited the expected behavior for the cell line maintained in this manner and that resistance to the effects of ADM was approximately 10 times that of the ADM-sensitive UV-2237RR. The plating efficiency of all 3 independently derived hybrid clones was midway between these 2 phenotypes, suggesting that ADM resistance is an incompletely dominant trait. The hybrid line derived from the fusion of the UV-2237 parent line and the UV-2237RR line (UV-2237RR x UV-2237 parent) was no more resistant to ADM than were the 2 individual lines.

**Uptake and Efflux Studies.** Results from a typical uptake experiment are presented in Chart 6. Identical curves were obtained whether the [14C]ADM was added at a concentration of 2 or 20 μg/ml. In the first 10 min, uptake by the UV-2237-ADM R cells was very similar to that by UV-2237 cells. However, the resistant line rapidly achieved a near-plateau of incorporation such that, by 60 min, there was a 3-fold and by 120 min a 6-fold difference in incorporation between the 2 cell lines (Chart 6). Efflux experiments consistently showed a difference in the
rates of efflux of the radiolabeled drug, although these differences were not always as large as those obtained in the experiment illustrated in Chart 7. The UV-2237-ADM line was better able to actively export the [14C]ADM than was the more sensitive UV-2237 line.

DISCUSSION

We have isolated and propagated in vitro a variant line of the murine UV-2237 fibrosarcoma that is resistant to the cytotoxic action of ADM. This resistance is maintained in the absence of ADM, whether the cells are grown in vitro or in vivo. Cells of the UV-2237-ADM line appeared normal when maintained in 1.0 µg ADM per ml (Fig. 1), whereas exposure of UV-2237 parent cells to the same levels of ADM for less than 48 hr produced such morphological changes as increased size and development of filamentous projections, comparable to changes described in other tumor systems maintained under similar conditions (30).

The development of clones or cell lines that are resistant to drugs, including ADM, is a frequent complication of chemotherapy (12). Studies of such resistance have focused on the differences between sensitive and resistant variants isolated from the same parent line of mammalian cells. Until now, most such variants have been isolated from the Chinese hamster ovary cell line (17), from leukemias (22, 28, 38), or from ascites-grown tumor lines (8, 37). Apart from brief mentions of a murine neuroblastoma (2) and a murine sarcoma (34), there are no other reports on the isolation of such variants from a solid tumor line. Resistance to ADM is often associated with decreased drug uptake (8, 14, 16, 29), which may be related to alterations in permeability of the cell membrane (32) or glycoprotein content of the cell (15) or may be the consequence of increased active efflux of the drug from the cell (18, 19).

The UV-2237-ADM line exhibited cross-resistance to a wide range of chemotherapeutic agents with dissimilar chemical structures and mechanisms of action; these agents include ACT D, AD 32, AMSA, DNR, MIT C, VBL, and VCR (Chart 4). Reciprocal cross-resistance between ADM and DNR, which bears a hydrogen atom in the acetyl moiety instead of a hydroxyl group, has been well described in experimental tumors (7, 9, 19, 21, 32, 37). Cross-resistance has also been reported with AD 32 (21), an N-acetylated derivative, although AD 32 does not bind to DNA (33), unlike the other anthracycline derivatives. Cross-resistance to the Vinca alkaloids (2, 7, 21) and the nonanthracycline DNA-intercalating agents ACT D and AMSA (3, 21, 39) has also been associated with ADM-resistant lines. In contrast to several other studies (21, 31), however, we found cross-resistance to the alkylating agent MIT C. The basis for this observed cross-resistance is, at the present time, unknown. This type of pleiotropic cross-resistance has been associated with membrane alterations resulting in reduced permeability (1). Indeed, Ling and his colleagues have shown that Chinese hamster ovary cells selected for resistance to colchicine have a drastic reduction in permeability to not only that drug but also a number of apparently unrelated agents (25–27). Therefore, a membrane mutation may be responsible for the resistance of the UV-2237-ADM line to some drugs. However, this is unlikely to be the total explanation. Pretreatment of the 2 cell lines with the metabolic inhibitor sodium azide leads to equal uptake of [14C]ADM, suggesting that passive diffusion alone is not sufficient to account for the observed differences between UV-2237 and UV-2237-ADM lines. Equally, the efflux experiments indicate that the UV-2237-ADM cells are able to actively export ADM at a faster rate than are the sensitive UV-2237 cells. These findings are consistent with observations made earlier by other workers who used different cell lines (18, 19). We are currently investigating the exact basis of the resistance exhibited by the UV-2237-ADM line. ADM binds and intercalates with double-stranded DNA with relatively high efficiency, and many investigators agree that the cytotoxic activity of this drug is mediated through this interaction (13, 23, 36). A recent report has suggested that some of the cytotoxic activity of ADM may be a consequence of interactions at the cell surface without any necessity for the drug to enter the cell (35). This possibility raises a number of intriguing questions regarding the nature of reported differences in the cell surfaces of sensitive and resistant variants (15).

Results from the cell-to-cell hybridization studies showed that the ADM resistance is an incompletely dominant trait (Chart 5). Cell-to-cell fusion may occur in solid tumors, possibly between cells of differing malignant capacity, and may give rise to hybrids that constitute a small but important component of the tumor cell population (20). Drug resistance in different clones of tumor cells may possibly arise in this manner, either by selection of...
preexisting clones or as a result of in situ hybridization. The stability of the ADM-resistant characteristic in vivo and the possession of a "universal fuser" line of UV-2237 should allow us to further explore this possibility in vivo.

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REFERENCES

Fig. 1. a, UV-2237 parent cells maintained on plastic in CMEM; b, UV-2237-ADM<sup>®</sup> cells maintained on plastic in CMEM containing ADM (1 μg/ml).
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