Comparative Survival in Tissue Culture of Normal and Neoplastic Human Cells Exposed to Adriamycin

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SUMMARY

In an effort to develop an in vitro predictive test for the clinical usefulness of adriamycin, we have grown human cells derived from normal tissue (skin, muscle, and Girardi heart cells) and human neoplastic cells in tissue culture and exposed them to varying concentrations of adriamycin for 1, 24, 48, and 72 hr. Cardiac cells were extremely sensitive to adriamycin; lesser degrees of inhibition were seen in order of decreasing sensitivity by sarcoma and melanoma cells, normal muscle, and normal skin fibroblasts. The relative sensitivity of tissues correlated in a general way with clinical experience; however, the in vitro test results in two patients treated with adriamycin did not correlate with their clinical course. Although our findings in vitro were not useful clinically, we suggest that further study of human cardiac cells in tissue culture may improve our understanding of adriamycin cardiotoxicity.

INTRODUCTION

Adriamycin (NSC 123127) is an antitumor antibiotic of the anthracycline group isolated from cultures of Streptomyces peucetius var. caesius. It is useful in the therapy of a variety of human tumors, particularly soft-tissue sarcomas, malignant lymphomas, and carcinomas of the breast and thyroid (4). Unfortunately, it is also a toxic drug which can cause hematopoietic dysfunctions, alopecia, stomatitis, and a poorly understood form of cardiac dysfunction. Cardiac damage may lead to fatal congestive heart failure, which has been most common in patients receiving total doses of adriamycin exceeding 550 mg/sq m (4, 13). Ideally, one would prefer to reserve this toxic drug for patients with tumors known to be biochemically sensitive to its cytotoxic effects at clinically tolerable drug concentrations. In an effort to develop an in vitro test for the prediction of clinical response to adriamycin, we have grown human normal cells and neoplastic cells in tissue culture and exposed them to adriamycin at varying concentrations for 1 to 72 hr. This report summarizes our experience with 3 cell lines from human skin, 2 lines from human muscle, 6 human melanoma lines, 3 sarcoma lines, and the Girardi human heart line.

MATERIALS AND METHODS

Chemicals. Adriamycin (14-hydroxydaunorubicin, NSC 123127, from Farmitalia, Milan, Italy) solutions of 1 mg/ml in 0.9% NaCl solution were prepared and diluted with growth medium just prior to use.

Cell Cultures. Girardi human heart cells were purchased from Microbiological Associates, Inc., Bethesda, Md. A detailed description of the biological characteristics of this cell line has been published (13). This cell line was initially established in 1956 from the right atrial appendage of a 41-year-old male. Initially, the cells grew as fibroblasts and, after 4.5 months, became epithelial-like. The cell line has been of value for the preparation of measles antigen and provides a sensitive assay culture for measles virus infectivity and isolation. The line also supports a variety of other viruses. Previous studies have shown that it produces tumors in the cheek pouch of the Syrian hamster with $10^6$ cells without cortisone, and with $10^8$ cells with cortisone. A culture in approximately the 500th serial passage was submitted to the Animal Cell Culture Collection in February 1964. The cell line did not contain the HSL antigen common to all strains of HeLa cells tested. Chromosome analysis of this cell reveals 46 chromosomes with 3 marker chromosomes. It retains its epithelial character and has a plating efficiency of approximately 60% in Eagle’s minimal essential medium with 10% calf serum.

Early-passage cell lines of normal human skin and muscle, human malignant melanoma, and soft-tissue sarcomas were used between Passages 1 and 25 for this study. These cell lines were originally established from primary tumor specimens in the laboratories of Dr. D. L. Morton of the Department of Surgery at the University of California, Los Angeles, Calif. Detailed studies of these cell lines are in progress and will be published separately. The established lines were maintained in Falcon T-75 No. 3024 flasks containing growth medium (Roswell Park Memorial Institute 1640) supplemented with 10% fetal calf serum, glutamine (0.3 mg/ml), gentamicin (25 µg/ml), and amphotericin B (10 µg/ml). The average doubling time of all cells was less than 48 hr. Cells were kept in exponential growth with feeding twice a week and passing at confluency. All cultures were tested independently and were...
found to be free of contamination by Mycoplasma (11).

Microinhibition Method. Cells in exponential growth were detached from Falcon T-75 flasks with 0.1% EDTA at pH 7.0. Each cell suspension was washed, resuspended in growth medium, and adjusted to plate 100 to 150 cells/well in Terasaki plates (microtest tissue-culture plate; Falcon Plastic 3034). After 24 hr of incubation in 5% CO2 at 37°, the plates were washed and refed with fresh media.

A different concentration of adriamycin was added to each well (0.001 to 10 μg/ml in 10 μl of medium). Each cell line was exposed to adriamycin on a separate Terasaki plate for 1-, 24-, 48-, and 72-hr periods. After each drug-exposure period, the treated plate was washed and refed with fresh medium for a 24-hr period. Plates were then fixed with 100% cold methanol and stained with Giesma, and the cells were counted. The criteria for cellular integrity included cell attachment, an intact nucleus, and an intact cell membrane.

In order to determine the total cell counts representing viable cells, we devised a modification of the above-described method to count cells with trypan blue (Grand Island Biological Co., Santa Clara, Calif.). Tissue culture Petri dishes (60 x 15 mm) were plated with 5 X 10^5 cells in 3 ml and incubated 24 hr (37°, 5% CO2). Each culture dish was washed with fresh medium. Adriamycin dose dilutions of 0.01, 0.10, and 1.0 μg/ml were added for 24- and 48-hr periods. After drug exposure, each monolayer culture was washed and refed with fresh medium for 24 hr. The cells were then detached with 0.1% EDTA at pH 8.0 with the use of a sterile 1-ml syringe to scrape and aspirate cells to form a single cell suspension. Aliquots were removed for trypan blue exclusion count.

The inhibitory index was calculated as follows: (a) determine the average number of cells/control well, (b) subtract the average number of cells/experimental well and, (c) divide the latter number by the average number of cells in the control wells. The ID_{50}^3 equals that concentration of adriamycin in μg/ml that will inhibit cell growth by 50%, as shown by an inhibitory index of 0.5.

RESULTS

Twenty-nine experiments were performed utilizing our standard assay in 15 normal or neoplastic cell lines. In each experiment, a clear-cut dose-response curve could be defined, with low doses and short exposure times resulting in less inhibition than was seen at higher doses or longer exposures. An inhibitory index was calculated for every dose and time of exposure, and these were plotted on graph paper as either the mean inhibitory index for a group of replicate experiments. Chart 1 illustrates this analysis as it applies to 4 separate experiments with the Girardi human heart line. Similar curves were seen with the other normal and neoplastic cell lines tested, and in each case it was possible to identify clearly the dose of adriamycin that inhibited 50% of the cells in question (ID_{50}).

We have compared the reproducibility of our morphologically based standard assay, with the same assay supplemented by trypan blue. Table 1 demonstrates the ID_{50} for adriamycin seen at 24 hr in 5 human malignant melanoma lines tested with and without trypan blue exposure. There is no statistical difference between these sets of paired data [p = 0.25 (7)].

The mean adriamycin ID_{50} at 1, 24, 48, and 72 hr of drug exposure for each type of cell line tested is shown in Table 2. The data are organized to demonstrate the spectrum of drug sensitivity from the most- to the least-sensitive group. Clearly, the Girardi heart cells were by far the most sensitive line tested, whereas muscle and skin fibroblasts were distinctly less sensitive. It is possible to directly compare these in vitro data with the results of adriamycin treatment in 2 patients, and to indirectly compare results with what is known about the adriamycin sensitivity of 2 tissues. Specifically, 1 patient with melanoma provided melanoma cells for study and also received adriamycin. There was no clinical antitumor response. The in vitro adriamycin ID_{50} for this melanoma cell line at 24 and 48 hr was 0.05 and 0.02 μg/ml, respectively. One patient with a soft-tissue sarcoma received adriamycin and enjoyed a good clinical response. The adriamycin ID_{50} for cells derived from

<table>
<thead>
<tr>
<th>Patient</th>
<th>Morphological assay</th>
<th>Trypan blue assay</th>
</tr>
</thead>
<tbody>
<tr>
<td>E. D.</td>
<td>0.05</td>
<td>0.05</td>
</tr>
<tr>
<td>R. O.</td>
<td>0.05</td>
<td>0.05</td>
</tr>
<tr>
<td>R. B.</td>
<td>0.12</td>
<td>0.04</td>
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<tr>
<td>K. O.</td>
<td>0.08</td>
<td>0.01</td>
</tr>
<tr>
<td>D. A.</td>
<td>0.08</td>
<td>0.10</td>
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The abbreviation used is: ID_{50}, the dose of drug that inhibits cell growth by 50%.
this sarcoma was 0.082 μg/ml at 24 hr and 0.035 μg/ml at 48 hr. Indirect comparisons of in vivo and in vitro adriamycin effects are possible for skin and muscle cells, since it is known that these tissues are clinically resistant to adriamycin damage in nearly all patients.

**DISCUSSION**

Physicians routinely use laboratory screening tests to select the appropriate antibiotic for the treatment of a patient with a bacterial infection. Since the start of the modern era of cancer chemotherapy in the 1940's, there have been analogous attempts to develop such tests to aid the physician in the treatment of cancer (5). If the site of action of a chemotherapeutic drug is known, the effect of the agent on a specific enzyme or metabolic pathway in isolated tumor cells may be compared with its clinical effectiveness. Although it is known that adriamycin binds avidly to DNA (6), this characteristic has not been sufficiently unique to allow the development of a specific assay to predict the response of treated tissues in patients with cancer. For drugs such as adriamycin, for which an assessment of primary drug action is not presently feasible, other less direct approaches to defining drug sensitivity have been attempted. Many secondary drug effects have been examined in human cells for drugs other than adriamycin, including effects on oxygen consumption, carbohydrate metabolism, nucleic acid synthesis, and exclusion of supravital stains. Certain minimal conditions are needed for all such tests (5), and we were encouraged to proceed, since our test system and drug of primary interest appeared to satisfy the following requirements.

1. The drug must be active in the form in which it is added to the in vitro system or must be converted to an active form by the constituents of that system. This criterion appears to be met for adriamycin by virtue of the profound effect of the unchanged drug on human chromosomes in vitro (14), its powerful lethal effects against HeLa cells and human lymphoma in culture (8, 12), and its in vitro effect against Chinese hamster cells (2). However, adriamycin in humans does undergo metabolic alterations (3).

2. The metabolism of the malignant cells in vivo and in vitro must be sufficiently similar so that drug effects under the 2 conditions are comparable. This condition is difficult to prove, although there are data to support the general approach utilized in this investigation (1, 9).

3. There must be sufficient time for drug action to become manifest. This criterion is fulfilled, in that our drug-exposure times are comparable to those seen in vivo with clinically useful schedules of drug administration (4).

4. A representative sample of tumor must be obtained for testing. This criterion is difficult to prove for any investigation of this type. However, all of our cell lines were started from well-defined normal or neoplastic tissues, and they retained a morphological similarity to their tissue or origin.

In view of these requirements, it should not be surprising that in vitro tests in general have been either unreliable or too cumbersome for routine use. The situation is compounded by the heterogeneous cell populations composing most human solid tumors, as well as by the fact that true biochemical sensitivity may exist to a given drug, and yet tumor regression may not occur when the drug is given to the patient. Tumor cells may be killed, but their destruction may be masked in at least 2 ways: killed, sensitive cells may be replaced by fibrous tissue, or drug-resistant cells present in the original tumor may rapidly assume dominance when the sensitive cells are killed. In any case, significant tumor response to drug may escape clinical detection.

In view of these problems, one must not ask too much of predictive systems for anticancer drugs. The bacteriologist asks his in vitro test to answer only 2 questions: (a) will the drug kill bacteria? and (b) at what concentrations is it effective? Similarly, the cancer chemotherapist can, at best, hope to gain information about dose-dependent cytotoxicity for malignant cells (5). This very limited goal was certainly realized in this study; however, in the limited situation in which a correlation could be made with the response to adriamycin therapy in vivo and in vitro, the responsive tumor line in vivo was less responsive in vitro than the resistant cell line. Thus, we were unable to confirm a clinically useful role for our particular assay as an in vitro-predictive test.

Despite our failure to develop a clinically useful, in vitro predictive test for adriamycin, we were intrigued by our results with the Girardi human heart line. Although we suspect that this cell line may have undergone malignant transformation, we believe that this and other cardiac cell lines deserve further study. Certainly, the Girardi cell line was exquisitely sensitive to adriamycin. It would be of great interest to define the biochemical basis for this extreme sensitivity. It would be of particular interest to compare normal muscle cells and the Girardi heart cell line in the following areas: (a) relative drug
uptake (transport), (b) relative binding of adriamycin to DNA, (c) relative inhibition of RNA, DNA, and protein synthesis, and (d) relative toxicity of a variety of newly described adriamycin metabolites.

ACKNOWLEDGMENTS

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