Cytotoxic Efficacy of Reconstituted and Stored Antitumor Agents

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ABSTRACT

The stability of the cell-killing efficacy of 10 antitumor drugs stored at different temperatures and concentrations was evaluated on an established human colon carcinoma cell line using the colony formation technique. Drugs were reconstituted in the appropriate solvent, further diluted in 0.9% NaCl solution to obtain the desired storage concentration, and stored at 4 °C, −20 °C, and −70 °C for periods of up to 3 weeks. Cytotoxic efficacy was tested weekly by exposing the cells to the final drug dilution for 1 h at 37 °C.

Stability of cytotoxic potency depended on the particular drug, and the duration, concentration, and temperature of storage but, in general, most drugs remained efficacious only within limited periods of time (at most, 2 weeks). These results suggest that the conditions of drug storage following reconstitution are critical factors with respect to lethal efficacy. Therefore, analysis of in vitro cytotoxic efficacy can be meaningful only when storage conditions are specified in order to avoid false-negative results in the human tumor clonogenic assay.

INTRODUCTION

The human tumor clonogenic assay of Hamburger and Salmon (6) offers potential improvement in the individualized treatment of the cancer patient (9, 12, 17). However, the vagaries of some of the conclusions and the large discrepancies in results among different investigators (16) have prompted cautionary reports against its widespread application in clinical practice (15, 18). Many aspects of the assay still require technical improvement and standardization. Several of these aspects have been or are being resolved (14), including the normalization of tumor cell sensitivity using the response of bone marrow granulocyte-macrophage progenitor cells as a frame of reference (8).

An important source of variability develops during the preparatory stages of the assay (12, 14) and may include the possibility of nonuniformity of killing efficacy of the antitumor agent used in the study. Some reports have recently addressed the question of biological stability after the drug was admixed with the semi-solid support matrix (7, 10), and one investigation has presented the problems involved in the preparation (i.e., solution and filtration) of the drug (11). Because very few laboratories use freshly prepared drug solutions, loss of the original potency after reconstitution and storage is another potential source of error. Most investigators dilute the antitumor agents to convenient concentrations for the different drug concentrations were normalized with respect to the individual controls for each experiment. All experiments were repeated at least twice with triplicate samples for each drug (9, 12, 17). These preparations are then thawed after various subsequent intervals and used in the assay under the undocumented assumption of retained potency.

We have previously reported in preliminary form that some agents may not maintain a consistent degree of cell-killing efficiency (19). This report formalizes this contention and demonstrates that most agents lose significant activity when stored in diluted form, a circumstance that may lead to severe discrepancies in the assessment of drug sensitivities as the material ages in storage.

MATERIALS AND METHODS

Cell Line. The cells used in this investigation were from the carcinoma embryonic antigen-producing colon carcinoma line LoVo (3). Cells are maintained as monolayer cultures in Ham's F-10 medium supplemented with 20% fetal calf serum, vitamins, glutamine, and antibiotics. The doubling time of exponentially growing cells is 37 h, and the generation time is 29 h (5); in this series of experiments, the plating efficiency ranged from 47 to 73%.

Drugs. Ten commonly used antitumor drugs (listed in Table 1) were selected for this investigation and reconstituted according to the manufacturers' instructions. An aliquot of this original solution was stored without the further manipulations described below. Further dilutions were made in 0.9% NaCl solution (saline) to obtain the desired storage concentrations (3-fold the final treatment concentration) in a volume of 1 ml. These solutions were either used immediately or stored at 3 different temperatures (4 °C, −20 °C, and −70 °C) in tubes made of siliconized glass (Borosilicate T-1209-3; American Scientific Products, Houston, TX) or plastic (polystyrene tubes, Falcon No. 2054; Curity-Matheson Scientific, Houston, TX) (1). Tubes were wrapped with aluminum foil to protect the contents from light. For cell treatment, drugs were further diluted in medium to attain the desired treatment concentration, and the pH was adjusted, if necessary, to 7.2 to 7.4.

Cell Survival Assay. Stock cultures were harvested by standard procedures reported previously (3) and counted with the aid of a Coulter Counter Model ZBI electronic particle counter (Coulter Electronics, Inc., Hialeah, FL). Cell suspension aliquots were seeded into 60-mm Petri dishes (5 × 10^6 cells/dish) and incubated at 37 °C in a 5% CO₂ atmosphere in air for 48 to 72 h to achieve exponential growth. The medium was discarded, and the cells were exposed to the desired drug concentrations for exactly 1 h at 37 °C. The drug was then decanted, and the cells were washed twice in saline, harvested as a monodispersed suspension, and counted. Known aliquots of each cell suspension were dispensed into 60-mm Petri dishes so that 50 to 100 colonies would appear after a 21-day incubation in a 5% CO₂ humidified atmosphere at 37 °C. The colonies were stained with 2% crystal violet in 95% ethanol and scored under a stereomicroscope. Viability was defined as the ability of single cells to give rise to a colony of ≥50 cells. In each experiment, the plating efficiency of at least 6 control cultures was assessed simultaneously. Control cultures consisted of cells treated in exactly the same manner as the test cells but without receiving the drug. The survival fractions for the different drug concentrations were normalized with respect to the individual controls for each experiment. All experiments were repeated at least twice with triplicate samples for each drug.
CYTOTOXIC ACTIVITY OF STORED ANTICANCER DRUGS

List of antitumor agents used for evaluation of lethal stability

<table>
<thead>
<tr>
<th>Common name and abbreviation</th>
<th>Chemical name</th>
<th>NSC No.</th>
<th>Solvent used for reconstitution</th>
<th>Storage concentrations</th>
<th>Material used for storage</th>
<th>Manufacturer (location)</th>
</tr>
</thead>
<tbody>
<tr>
<td>Adriamycin (ADR)</td>
<td>1,4-Hydroxydaunorubicin</td>
<td>123127</td>
<td>0.9% NaCl solution</td>
<td>0.60 μg/ml, 1.50 μg/ml, 2.00 mg/ml</td>
<td>Plastic</td>
<td>Farmitalia Carlo Erba (Italy)</td>
</tr>
<tr>
<td>Carmustine (BCNU)</td>
<td>1,3-Bis(2-chloroethyl)-1-nitrosourea</td>
<td>409962</td>
<td>Absolute ethanol</td>
<td>0.00 μg/ml, 0.00 μg/ml, 10.00 mg/ml</td>
<td>Glass</td>
<td>Bristol (Syracuse, NY)</td>
</tr>
<tr>
<td>Bleomycin (BLEO)</td>
<td>Bleomycin sulfate</td>
<td>125066</td>
<td>0.9% NaCl solution</td>
<td>6.00 μg/ml, 30.00 μg/ml, 3.00 mg/ml</td>
<td>Glass</td>
<td>Bristol</td>
</tr>
<tr>
<td>cis-Diaminedichloroplatinum (II)</td>
<td>cis-diaminedichloroplatinum (II)</td>
<td>119875</td>
<td>H2O</td>
<td>15.00 μg/ml, 30.00 μg/ml, 1.00 mg/ml</td>
<td>Glass</td>
<td>Bristol</td>
</tr>
<tr>
<td>Etoposide (VPS)</td>
<td>4'-Demethylepipodophytoxin 9(4,6-O-ethylidene-β-O-glucopyranoside)</td>
<td>141540</td>
<td>0.9% NaCl solution</td>
<td>6.00 μg/ml, 15.00 μg/ml, 20.00 mg/ml</td>
<td>Glass</td>
<td>Bristol</td>
</tr>
<tr>
<td>5-Fluorouracil (5-FUra)</td>
<td>5-Fluorouracil</td>
<td>19893</td>
<td>36% HCl in ethanol followed by 12 ml Na2PO4 and 0.6 ml propylene glycol/ml of H2O</td>
<td>2.40 μg/ml, 3.75 μg/ml, 10.00 mg/ml</td>
<td>Glass</td>
<td>National Cancer Institute (Bethesda, MD)</td>
</tr>
<tr>
<td>Melphalan (L-PAM)</td>
<td>Phenylalanine mustard</td>
<td>8806</td>
<td>36% HCl in ethanol followed by 12 ml Na2PO4 and 0.6 ml propylene glycol/ml of H2O</td>
<td>2.40 μg/ml, 3.75 μg/ml, 10.00 mg/ml</td>
<td>Glass</td>
<td>National Cancer Institute (Bethesda, MD)</td>
</tr>
<tr>
<td>Mitoxantrone</td>
<td>1,4-Dihydroxy-5,8-bis[2-(2-hydroxyethylamino)-9,10-anthracenedione dihydrochloride</td>
<td>301739</td>
<td>0.30 μg/ml, 0.75 μg/ml, 2.00 mg/ml</td>
<td>3.00 μg/ml, 6.00 μg/ml, 50.00 mg/ml</td>
<td>Plastic</td>
<td>Taylor Pharmaceutical (Decatur, IL)</td>
</tr>
<tr>
<td>Mitomycin C (Mito C)</td>
<td>Deacetyl vinblastine amide sulfate</td>
<td>245467</td>
<td>0.9% NaCl and 0.9% benzyl alcohol in H2O</td>
<td>60.00 μg/ml, 225.00 μg/ml, 1.00 mg/ml</td>
<td>Glass</td>
<td>Eli Lilly (Indianapolis, IN)</td>
</tr>
<tr>
<td>Vindesine (VDS)</td>
<td>Deacetyl vinblastine amide sulfate</td>
<td>245467</td>
<td>0.9% NaCl and 0.9% benzyl alcohol in H2O</td>
<td>60.00 μg/ml, 225.00 μg/ml, 1.00 mg/ml</td>
<td>Glass</td>
<td>Eli Lilly</td>
</tr>
</tbody>
</table>

Experimental Design. At each time point, cells were treated with 2 separate concentrations of each drug; these concentrations were estimated to reduce survival by 40 to 60% and by 80 to 95% from previous experiments (2). To define the native lethal efficacy, cells were treated with dilutions of freshly prepared drug immediately after separating the aliquots for storage, and with dilutions of additional freshly reconstituted drug prepared at weekly intervals. This maneuver was intended to account for possible weekly variations in cell sensitivity when calculating the overall efficacy of the freshly prepared drug solution. At weekly intervals, and for 3 consecutive weeks following the initial determination of cytotoxic efficacy, new stock cells were harvested, seeded into Petri dishes, incubated to logarithmic growth as described above, and treated with 2 concentrations of each drug. The drug used in the experiment was either freshly prepared or was briefly warmed up to 37 °C after removing from storage. All dilutions to treatment concentration of the freshly prepared or stored drugs were made in medium at 37 °C. During the last week of the experimental interval, cells were also treated with dilutions of the originally reconstituted drug concentration (undiluted) that had been stored under the same conditions as the test concentrations.

RESULTS

Only 3 drugs, mitomycin C, etoposide, and bleomycin, showed a consistent cell killing efficacy over the 3-week interval regardless of the storage temperature and concentration (Chart 1). However, for low concentrations of bleomycin stored at 4 °C, cytotoxic activity declined after 2 weeks and disappeared completely after 3 weeks. For all 3 drugs, storage at the originally reconstituted concentration retained the degree of cell kill only at the 80 to 95% level but displayed some variations at the 40 to 60% cell kill level.

For VDS and BCNU, storage at a low concentration (used for the 40 to 60% cell kill) resulted in rapid deterioration of cytotoxic activity beginning after 1 week, regardless of storage temperature (Chart 2). After the third week, the lethal efficacy of the drugs stored at this concentration decreased to almost zero. The decline pattern of the lethal efficacy of Adriamycin was similar to those of VDS and BCNU, but the decrease of its cytotoxicity was slower and to a lesser degree. Storage at the higher concentrations (80 to 90% cell kill) retained the lethal activity throughout the experimental interval for all 3 drugs. Storage at the original reconstitution concentration retained cytotoxic activity at both cell killing levels.

For cis-DDP, mitoxantrone, 5-FUra, and L-PAM, storage at both working concentrations decreased cytotoxic activity regard-
less of temperature; efficacy declined by greater than 50% after 3 weeks of storage (Chart 3). In contrast, and with the exception of L-PAM, storage at the reconstitution concentrations retained the magnitude of the cytotoxic activity at the level similar to that of freshly prepared drug.

DISCUSSION

We evaluated the stability of the lethal efficacy of 10 stored antitumor drugs commonly used for the human tumor cloning assay. As target, we used a well-characterized human colon carcinoma cell line that provides consistent survival responses when tested against these antitumor drugs. The cell line requires rather simple manipulations to obtain a viable mononuclear suspension (3) and yields large colonies with a high plating efficiency (4), properties that reduced the number of variables (i.e., cell disaggregation, cell clumps, etc.) that could possibly interfere with the uniformity of results. To ensure further reproducibility and to decrease biological variability with respect to analytical error, we also tested cell response against freshly prepared drugs on a weekly basis. The combined results provided the frame of reference for comparisons with the survival response to stored drug dilutions. We elected to use not the pure drug,
but the antitumor agent formulated for clinical use (with buffers, carriers, preservatives, etc.), in order to imitate common laboratory practice. Finally, drug solutions were prepared steriley to avoid filtration procedures that may retain significant amounts of drug (11).

Our results demonstrate that very few agents retain their native cytotoxic potency when stored under refrigerated or frozen conditions at low stock concentrations. Thus, only etoposide, bleomycin, and mitomycin C were as effective 3 weeks after solvation and storage as the freshly prepared drug. Even then, there was an obvious although still statistically insignificant trend toward decreased efficacy and, in the case of low bleomycin concentrations stored at 4 °C, a complete loss of potency. For 3 other drugs, VDS, Adriamycin, and BCNU, almost complete loss of efficacy occurred at the low storage concentrations regardless of temperature, even as early as 1 week (VDS and BCNU) after reconstitution. The other 4 drugs (L-PAM, cis-DDP, mitoxantrone, and 5-FUra) either lost significant degrees of efficacy or were totally ineffective after 2 to 3 weeks of storage regardless of temperature. Only when drugs were stored at very high concentrations did the solution retain its native potency.

Although storing drug solutions in the frozen state is a traditional procedure, our results indicate that the procedure may lead to decreased biological efficacy. For some drugs (i.e., 5-FUra and cis-DDP), this may result from precipitation with incomplete resolution, and for this reason room temperature is the recommended storage temperature for these agents. For other drugs, loss of potency may be ascribed to a degradative interaction with the additives supplied in the clinical formulation, an interaction that may occur even in the frozen state; this situation apparently does not take place or appears to be insignificant if the mixture is prepared at the very high drug concentrations obtained in the original reconstituted solution.

Whatever the reason, our results demonstrate that reconstituted clinically formulated antitumor drugs gradually lose potency in storage regardless of temperature, especially if prepared at low working concentrations. This loss is particularly appreciable at the 40 to 60% level of cell kill, the range of activity for most
agents used in the Hamburger-Salmon assay. Thus, it seems reasonable that, to avoid potentially large errors in classifying sensitivity to a given agent, it would be mandatory to use freshly prepared drug, especially when a clinically formulated drug is used. If storage is necessary, the stability of antitumor agents must be ascertained to ensure that potency is retained.

REFERENCES

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