Activation of Cyclophosphamide for in Vitro Testing of Cell Sensitivity

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ABSTRACT

Two methods are described for the activation of cyclophosphamide by a liver microsomal preparation. These procedures were applicable to an assay in vitro which tests the sensitivity of tumor cells to the drug. Satisfactory results were obtained either by pretreatment of the cyclophosphamide and removal of the microsomes before testing or by the somewhat simpler procedure of mixing drug, microsomes, and test cells for the assay. Microsome treatment of bleomycin gave a smaller increase in activity, and much smaller effects were seen on some other drugs.

INTRODUCTION

Testing of tumor cells in vitro for their sensitivity to chemotherapeutic agents offers promise of great benefits to patients. The widely used antitumor agent, cyclophosphamide, requires activation (4) before it induces its inhibitory effect. This activation can be accomplished in vivo by enzymatic oxidation by liver microsomes (6). For tests in vitro of the susceptibility of cells to cyclophosphamide, the activation requirement has been met by the use of serum and urine of cyclophosphamide-treated animals (4, 8) and by use of 4-hydroperoxy-cyclophosphamide, a derivative of cyclophosphamide (8). Cyclophosphamide has been reported to be activated in vitro by liver slices (8), homogenates (1, 4), and microsomes (2, 3). In developing a method for testing in vitro the sensitivity of human tumor cells to potential chemotherapeutic agents (7), we faced the problem of activation. We describe here a simple procedure for the activation of cyclophosphamide in the testing of the susceptibility of cells in vitro.

MATERIALS AND METHODS

The drugs tested were doxorubicin (Adriamycin; Adria Laboratories, Wilmington, Del.), bleomycin sulfate (Benoxane; Bristol Laboratories, Syracuse, N. Y.), N,N',N''-triethylene thiophosphoramide (Thio-TEPA; Lederle Laboratories, Pearl River, N. Y.), cyclophosphamide (Cytoxan; Mead Johnson and Co., Evansville, Ind.), 5-fluorouracil (Roche Laboratories, Nutley, N. J.), and cis-diaminedichloroplatinum (cis-platinum; from the Division of Cancer Treatment, NIH, Bethesda, Md.). [methyl-3H]Thymidine (56.9 Ci/mmol), [3H]uridine (41.3 Ci/mmol) were purchased from New England Nuclear. The scintillation fluid for radioisotope determination consisted of 4 g of PPO and 0.1 g of POPOP per liter of toluene.

Culture medium was Trowell's T8 supplemented with 20% fetal bovine serum, streptomycin sulfate (50 μg/ml), penicillin G (62.5 units/ml), and Fungizone (0.5 μg/ml). Collagenase type I was obtained from Worthington Biochemical Corp. Ficoll (M.W., approximately 400,000) was from Pharmacia Fine Chemicals. Hypaque (sodium diatrizoate) was a 50% (w/v) aqueous solution made by Winthrop Laboratories.

Procedures for preparation of cells from human tumors, their culture in vitro, and their testing for drug sensitivity were described in detail elsewhere (7).

Briefly, human tumors were placed in serum-free medium at the operating room immediately after surgical excision. Human tumors growing in athymic "nude" mice were handled similarly. Tumor pieces were cut into 1- to 2-mm fragments and were incubated overnight at 37° in 0.1 to 0.05% collagenase in serum-containing medium. The partially dispersed tumor cells were passed gently through an 80 mesh sterile tissue sieve (E-C Apparatus), sedimented, and washed with medium. A Ficol-Hypaque density gradient was used to separate viable and nonviable cells from the tumor cell suspension. The live-cell layer was gently aspirated and washed twice with medium. The resulting cell button was suspended in medium and adjusted to a concentration of 5 to 6 × 10⁵ cells/ml for dispensing into Linbro No. 1S-MVC-96-TC microtiter dishes.

The microtiter plates containing cells and medium, with or without added drug and/or microsome preparation, were incubated at 37° for 72 hr in a humidified atmosphere of 5% CO₂ in air. Fifty μl of medium containing 1.5 μCi of tritium-labeled thymidine, uridine, or amino acid mixture were then added to appropriate wells, giving a concentration of 7.5 μCi/ml and a volume of 0.2 ml/well. The precursor concentrations (μM) were: thymidine, 0.13; uridine, 0.18. Incubation was continued for 12 hr. Acid-precipitable radioactivity was then determined. The inhibition of incorporation is expressed as the difference in incorporation between drug-treated and control cells, divided by incorporation in control cells, stated as percentage.

Preparation of Mouse Liver Microsomes. Four- to 8-week-old NIH Swiss mice were fed sterilized standard laboratory chow and were given drinking water containing 0.1% sodium phenobarbital for 1 week prior to sacrifice. The mice were killed by cervical dislocation, and the livers removed into sterile, chilled Krebs-Ringer phosphate buffer, containing 0.129 M NaCl, 0.0052 M KCl, 0.0018 M CaCl₂, 0.0013 M MgSO₄, and 0.01 M sodium phosphate (pH 7.4). The livers were washed several times with the buffer to remove blood and blood clots and were homogenized for less than 1 min, using 2 ml of cold buffer per g wet tissue, in a Potter-Elvehjem tissue grinder. The homogenate was centrifuged at 27,000 × g for 10 min at 4°. The resulting pellet containing mitochondria and nuclei was discarded, and the supernatant fluid containing the micro-
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somes was centrifuged at 105,000 × g for 1 hr. The microsomal pellet was resuspended in the buffer and assayed for protein content (5). The microsomes were adjusted to 23 mg protein per ml, dispensed into 0.3-ml volumes, and frozen at −70° until use (1).

**Activation of Drugs with Liver Microsomes.** The microsomal incubation mixture contained 0.3 ml of adjusted microsomes (6.9 mg of protein), 5 mm glucose 6-phosphate, 4 mm NADP, and 100 mm sodium phosphate buffer (pH 7.4). The optimum concentrations of microsomes and cofactors were determined in preliminary experiments.

The activation of drugs by microsomal mixture was achieved by 2 different methods. In Method 1, 1 to 5 μl of microsomal mixture were added along with the tumor cells to control and experimental wells of the microtiter plate containing varying concentrations of drugs. The microsomes were allowed to activate the drug during the 72 hr of the assay.

In an alternative procedure, Method 2, the drugs were incubated with microsomal mixture for 60 min at 37° in a shaking water bath. The reaction was then terminated by chilling on ice. The microsomes were removed by centrifugation at 105,000 × g for 60 min, and the soluble fraction was used in the drug sensitivity assay.

**RESULTS**

Treatment of cyclophosphamide with an adequate level of liver microsomes and cofactors resulted in striking increases in the capacity of the drug to inhibit incorporation of the labeled precursors into acid-insoluble material. The experiment shown in Chart 1, using Method 1 and the incorporation of uridine into nude mouse-supported human bladder tumor cells as the indicator, showed that 0.1 μl of microsome suspension had little effect and that 5 μl gave the maximum activation. Amounts greater than 5 μl sometimes caused toxicity to cells in vitro. Since 3 μl of microsome suspension gave near-maximum activation and avoided the latter difficulty, it was adopted as the standard volume. The microsome suspension itself did not incorporate labeled precursors above background level (Table 1).

**DISCUSSION**

Activation of cyclophosphamide by liver microsomal enzymes resulted in a dramatic increase in its inhibition of incorporation of precursors of DNA, RNA, and protein into acid-insoluble material by human tumor cells in vitro (Charts 1 and 3). Occasional inhibition by cyclophosphamide in the absence of added microsomes, as with the cultured human rhabdo-

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**Table 1**

Incorporation of radioactively labeled metabolic precursors into acid-insoluble material by free microsomes

<table>
<thead>
<tr>
<th></th>
<th>3H-Amino acids (cpm)</th>
<th>3H-Thymidine (cpm)</th>
<th>3H-Uridine (cpm)</th>
</tr>
</thead>
<tbody>
<tr>
<td>5 μl microsomal mixture, 150 μl medium, 50 μl isotope</td>
<td>140</td>
<td>120</td>
<td>150</td>
</tr>
<tr>
<td>155 μl medium, 50 μl isotope</td>
<td>130</td>
<td>110</td>
<td>130</td>
</tr>
<tr>
<td>5 μl microsomal mixture, 6 × 10^4 cells, 150 μl medium, 50 μl isotope</td>
<td>53,000</td>
<td>43,000</td>
<td>66,000</td>
</tr>
</tbody>
</table>

* # Mean of 4 values.

* Rhabdomyosarcoma cell line (18574).

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**Chart 1**

The in vitro activation of cyclophosphamide by microsomal mixture measured by the inhibition of incorporation of uridine into acid-insoluble material. Cells used for the assay were from nude mouse-supported human bladder carcinoma (I-559). Cyclophosphamide amounts of 0.5, 2.0, and 5.0 μg gave final concentrations of 2.5, 10, and 25 μg/ml, respectively. [3H]Uridine concentration was 7.5 μCi/ml. Points, means of 4 experimental wells on the microtiter dish. Bars, S.E.; △, 0.1 μl of microsomal mixture per well; , 1 μl of microsomal mixture per well; O, 5 μl of microsomal mixture per well.

**Chart 2**

Comparison between 2 methods of activation of cyclophosphamide. The in vitro activation procedure is detailed under "Materials and Methods." Points, means of 4 experimental wells on the microtiter dish. Bars, S.E. Cells were from nude mouse-supported human carcinoma of the bladder (I-527). Isotope concentrations were 7.5 μCi/ml, and cyclophosphamide final concentrations were the same as in Chart 1. O, [3H-labeled amino acids); [3H-thymidine), preliminary microsome activation, followed by removal of microsomes before the assay (10 μl of microsomal mixture per 50 μl of cyclophosphamide).

Both Method 1 (microsomes present during treatment of the cells) and Method 2 (microsomes removed by centrifugation before cell incubation) were effective (Chart 2), although Method 2 required more of the microsomal suspension. Since Method 1 was more convenient and at least as effective, it was used as the standard procedure.

The effects of the presence of microsomes on the inhibitory activity of several chemotherapeutic drugs other than cyclophosphamide were also tested, and the results were shown in Chart 3. Presence of the microsomes did not interfere with the activity of any of the drugs tested. There was some apparent activation of some of the other drugs, notably bleomycin, at certain concentrations, but the effect was much less than with cyclophosphamide.
myosarcoma cells shown in Chart 3, may reflect a limited activating capacity of some test cell preparations. In all trials, however, the addition of liver microsomes substantially enhanced cyclophosphamide activity. Connors et al. (3) reported a similar effect of activating cyclophosphamide with liver microsomes in the treatment in vitro of cells from the Walker ascites tumor of the rat. Microsome activation reduced the required concentration of cyclophosphamide 20- to 40-fold in their rat cell system.

Inclusion of microsome suspension and cofactors caused a lesser enhancement of the activity of some other drugs (Chart 3), particularly bleomycin and possibly low doses of cis-platinum or Thio-TEPA. Yamanaka et al. (9) found an increase in bleomycin-induced strand breaks in DNA when a microsome preparation was incorporated in their system. While the amount of enhancement of activity did not seem to warrant routine use of a microsome preparation with drugs other than cyclophosphamide, we did not find any instances of microsome inhibition of drug activity; thus, this activation system may be useful with other agents.

For testing cell sensitivity to cyclophosphamide, activation of the drug by routine incorporation of a microsome preparation in the test system was clearly advantageous. The procedure was simple and convenient and used only small amounts of material.

REFERENCES
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