Activation and Inactivation of Cancer Chemotherapeutic Agents by Rat Hepatocytes Cocultured with Human Tumor Cell Lines

M. C. Alley, G. Powis, P. L. Appel, K. L. Kooistra, and M. M. Lieber


ABSTRACT

While colony formation assays provide sensitive indices of tumor cell proliferation and growth inhibition imposed by many chemotherapeutic agents, drugs which require metabolic activation lack activity in such assays. In the present study, we have utilized freshly isolated rat hepatocytes for the activation of drugs which are metabolized by hepatic microsomal as well as extramicrosomal enzymes. Hepatocytes in fluid medium are placed over soft-agarose matrix containing tumor-derived cells (e.g., A204, A549) within 35-mm culture dishes; drug and/or drug vehicle is added directly to the hepatocyte layer, and cultures are incubated for 24 hr prior to removal of the hepatocyte layer. Tumor cell colony formation is assessed following 7 to 10 days of incubation. Cyclophosphamide was used as a prototype agent to assess utility of the coculture methodology. In vitro treatment of rats with phenobarbital prior to hepatocyte isolation enhances cyclophosphamide toxicity in vitro, whereas pretreatment with carbon tetrachloride markedly reduced subsequent in vitro cyclophosphamide cytotoxicity. Hepatocyte:tumor cell cocultures provide an efficient means to detect metabolic activation and inactivation of several selected cancer chemotherapeutic agents as well. In the presence of hepatocytes, the 50% growth-inhibitory concentrations for cyclophosphamide, indicine N-oxide, and procarbazine are markedly decreased, whereas the 50% growth-inhibitory concentrations for [2,5-bis(1-aziridinyl)-3,6-diazio-1,4-cyclohexadiene-1,4-diy]bis(carbamic acid)diethyl ester, 1,3-bis-chloro(2-chloroethyl)-1-nitrosourea, dacarbazine, 5-fluorouracil, fltorafur, 1-(2-chloroethyl)-3-(4-methylcyclohexyl)-1-nitrosourea, and vincristine are significantly increased. By contrast, the 50% growth-inhibitory concentrations for actinomycin D, mitomycin C, 6-mercaptopurine, and other agents are unaffected by hepatocyte presence. Cryopreserved hepatocytes exhibit detectable levels of drug activation, although inadequate for routine use. Results suggest that hepatocyte:tumor cell cocultures may be well-suited for assessing the degree to which hepatic metabolism may activate or inactivate new anticancer drugs.

INTRODUCTION

A variety of tumor cell culture procedures are used to study mechanisms of anticancer drug action and in the search for new anticancer drugs. One such procedure, the SACFA,2 provides a sensitive index of tumor cell proliferation and is considered to be one of the best in vitro tests for antiproliferative drug activity (49, 59). However, one limitation of the conventional SACFA is that prodrugs which require metabolic activation lack activity in such systems. For example, preliminary results of a National Cancer Institute-sponsored trial of the SACFA failed to indicate activity for methotrexate, DTIC, procarbazine, hexamethylmelamine, and cyclophosphamide (26). While methotrexate is an antimetabolite, the activity of which may be antagonized by nutrients in the growth medium, the other drugs may require metabolic activation.

Use of cofactor-fortified hepatic microsomal fractions in conjunction with the SACFA has been described by others and ourselves (36, 40). While such liver preparations are suitable for incubation of tumor cells with drug prior to culture, they cannot be incorporated directly into the SACFA due to prominent inhibition of cell growth imposed by media used in preparing the microsomal fraction and supplemented cofactors. One further disadvantage of the microsomal activating system is that only prodrugs metabolized by cytochrome P-450, or a limited number of other microsomal enzymes, are activated. Enzymes in the liver other than cytochrome P-450 are known to activate drugs to form toxic metabolites, for example, sulfite, glucuronide, and acyl conjugations of N-hydroxyarylamine (5, 8, 31); glutathione conjugation of 1,2-dichloroethene (48); as well as metabolism of the anticancer drugs anilute mustard (14) and chlorambucil (17).

In contrast to fractionated liver homogenates, intact hepatocytes contain a full complement of enzymes necessary for drug activation or inactivation. Moreover, metabolites generated within intact hepatocytes are subject to restraints of compartmentalization and restricted transport similar to those operative in vivo. In this study, we report the utility of using rat hepatocytes as a drug-activating and -inactivating system in the SACFA.

MATERIALS AND METHODS

Human rhabdomyosarcoma (A204) and lung carcinoma (A549), continuous cell lines obtained from Frederick Cancer Research Center, Bethesda, MD, were maintained as bulk cell culture monolayers in multiple 75-sq cm flasks containing a standard growth medium: Dulbecco's modified Eagle's medium supplemented with 10% fetal calf serum; 100 units penicillin/ml; 100 μg streptomycin/ml; 2 mm l-glutamine; and 25 mm N-2-hydroxyethyl pipperazine-N'-2-ethane sulfonic acid (Grand Island Biological Co., Grand Island, NY). The medium was changed 3 times per week, and cells in exponential growth were passaged each week for a maximum of 15 weeks using medium containing 0.05% trypsin and 0.01% EDTA. Subsequent bulk cultures were reestablished from cell aliquots stored in liquid nitrogen. Each cell line was verified to be Mycoplasma free by culture (Virology Laboratory, Mayo Clinic).

Preliminary experiments were conducted with A204 cells growing on plastic surfaces. Cells in log-phase growth were plated at multiple densities such that final counts of between 100 and 200 colonies/60-mm culture dish were obtained following drug exposure (39). Culture dishes were placed in a 37° incubator for 24 hr to allow attachment of...
cells. Growth medium (5 ml) was replaced with warmed growth medium containing drug and hepatocytes as appropriate. After a predetermined time, culture dishes were rinsed 5 times with prewarmed growth medium to remove drug and then maintained in incubators at 37°. 5% CO₂:95% air, and 100% relative humidity for 10 days. Medium was removed, and dishes were washed with warmed 0.9% NaCl solution. Colonies were stained with 0.2% crystal violet in methanol for 10 min, rinsed with tap water, and counted manually. Quadruplicate culture dishes were used for each drug concentration.

All soft-agarose cultures were performed in similar fashion. Each 35-mm culture dish contained a base layer consisting of 0.5 ml of standard culture medium with 0.5% agarose (Seaplaque; FMC Corporation). On Day 0, cells in bulk culture were dissociated with trypsin and EDTA, washed once in growth medium, and subcultured by layering 1 × 10⁶ viable cells in 0.5 ml of growth medium with 0.3% agarose over each base layer. Cultures were examined with the aid of an inverted stage microscope, and only cultures containing uniformly distributed single-cell suspensions (<ten 30-μm diameter cell clusters and no 60-μm clusters) were accepted for subsequent evaluation. Cultures were maintained in cell culture incubators at 37°, 5% CO₂:95% air, and 100% relative humidity. On Day 1 (24 hr later), an upper layer of 1 ml of growth medium containing hepatocytes and/or drug was applied to each culture. After selected time intervals, hepatocytes were removed by aspiration, and agarose culture surfaces were washed twice with 0.5 ml of prewarmed growth medium and then overlaid with 1 ml of fresh growth medium. Colony formation was examined at daily intervals by conventional light microscopy. Cell lines formed a sufficient number of detectable colonies (≥60-μm diameter) for analysis following 7 to 12 days of incubation. Viable colonies were stained using a metabolizable tetrazolium salt, 2-(4-iodophenyl)-3-(4-nitrophenyl)-5-phenyltetrazolium chloride (Aldrich Chemical Co., Milwaukee, WI), and analyzed as described previously (1). Colony formation in studies with multiple hepatocyte densities was expressed as a relative to control cultures without drug or hepatocytes; colony formation in studies with multiple drug concentrations was expressed relative to control cultures containing hepatocytes at the same density but without drug.

Hepatic postmitochondrial fraction was prepared from livers of nonfasted Sprague-Dawley rats (Sprague-Dawley, Madison, WI) homogenized in 0.15 M KCl according to the method of Ernster et al. (16) and stored frozen at −196°. All cryopreserved hepatocytes were stored frozen at −196° with a cooling gradient of 3°/min from +4° to −4°, 1°/min from −4° to −10°, 0.3° to 0.5°/min from −10° to −40°, and 1° to 5°/min from −40° to −120°. All cryopreserved hepatocytes were stored frozen at −196° in liquid nitrogen for at least 1 week before thawing. Thawing was accomplished either rapidly by immersion of vials in a water bath at 37° or slowly through use of the CryoMed freezer (CryoMed, Mt. Clemens, MI) was used with a cooling gradient of 3°/min from +4° to −4°, 1°/min from −4° to −10°, 0.3° to 0.5°/min from −10° to −40°, and 1° to 5°/min from −40° to −120°. All cryopreserved hepatocytes were stored frozen at −196° in liquid nitrogen for at least 1 week before thawing. Thawing was accomplished either rapidly by immersion of vials in a water bath at 37° or slowly through use of the CryoMed freezer (0.5°/min). Dimethyl sulfoxide was removed from the thawed cells by washing the cells 3 times in growth medium. In some cases, α-tocopherol alcohol, 100 μg/ml, was added to the freezing medium, since work with hepatocytes in culture has shown it to prevent loss of hepatocyte functional capacity (21).

Cyclophosphamide was purchased from Sigma Chemical Co., St. Louis, MO. All other drugs* were supplied by the Drug Synthesis and Chemistry Branch, Division of Cancer Treatment, National Cancer Institute, Bethesda, MD.

Colony formation data were fitted to a monoeponential survival curve using the NONLIN nonlinear least-squares regression analysis program (41). Variance of the drug or prodrug concentration required to produce 50% inhibition of cell growth was obtained from the variance of the intercept and slope using a Taylor series expansion. Groups of data with and without hepatocytes were compared using Student's t test (53).

**RESULTS**

**Hepatic Postmitochondrial Fraction in SACFA.** Attempts to incorporate a cofactor-fortified hepatic postmitochondrial fraction into the SACFA were unsuccessful. Application of postmitochondrial fraction to soft-agar cultures for as little as 1 hr resulted in significant inhibition of subsequent A204 colony formation. This was traced to the 0.15 M KCl in which the postmitochondrial fraction was prepared. Using a more concentrated postmitochondrial fraction so that less KCl was applied to the SACFA, or using 0.25 M sucrose to prepare the postmitochondrial fraction, did not alleviate the problem of growth inhibition. Given the liver was homogenized in growth medium, which gave a very impure postmitochondrial fraction, acute inhibition of cell growth could be avoided, but when the cofactor-fortified postmitochondrial fraction was laid over the soft agar for 24 hr, the tumor cells grew slowly for 4 days and then stopped dividing. This problem was traced to the cofactor generating system, probably the glucose 6-phosphate.

**Hepatocytes and Cells Grown on Plastic Surfaces.** Several preliminary experiments with hepatocyte activating systems and cyclophosphamide as a prodrug were conducted using cells growing on plastic surfaces. Despite extensive precautions, it was not possible to isolate hepatocytes completely free of bacteria. However, by including gentamycin (100 μg/ml or 50 μg/ml) in the growth medium, bacterial contamination was obviated.

*BCNU; CCNU; MeCCNU; DTIC; 5-(3,3-dimethyl-1-triazeno)imidazole-4-carboxamide; N-isopropyl(2-methylhydrazino)-p-toluamide hydrochloride (procarbazine); indolinc N-oxide; 6-mercaptopurin; AZQ; 5-FU; (5-fluoro-1-tetrahydro-2-furyl)uracil (torafurul); mitomycin C; actinomycin D; 2,4,6-tris(dimethylaminomethylene)-5-(hexamethylenemine)-N-methylformamide; and 1,4,5,6,8-pentaazacacenaphthylen-3-amino-1,5-dihydro-5-methyl-1-(3-o-ribonucleosyl) (tricyclic nucleoside).
This concentration of antibiotics did not affect tumor cell growth or hepatocyte drug metabolism (used routinely by us in SACFA with primary human tumors). Studies were conducted with A204 cells grown on plastic surfaces with varying numbers of hepatocytes in the presence and absence of cyclophosphamide (Table 1). A problem encountered was that the A204 cells were not firmly fixed to the plastic surfaces, and some cells were lost when the surface of the culture was washed with growth medium to remove hepatocytes. The results show, however, that, while cyclophosphamide alone at 100 μg/ml exhibited no cytotoxicity towards A204 tumor cells, at the same drug concentration in the presence of hepatocytes, there was marked killing of tumor cells. All further studies were conducted in soft-agarose cell culture.

**Optimal Duration of Hepatocyte:A204 Cell Coculture.** The effect of hepatocyte-activated cyclophosphamide upon soft-agarose colony formation by A204 cells is summarized in Table 2. Cyclophosphamide at a concentration of 100 μg/ml in the absence of hepatocytes exhibited no significant cytotoxicity toward A204 cells. However, in the presence of hepatocytes for 24 hr, cyclophosphamide treatment resulted in significant growth inhibition. Coincubation of hepatocytes and cyclophosphamide for 1 hr provided insufficient drug activation. Exposure to hepatocytes alone for 7 days resulted in marked inhibition of A204 colony formation.

**Optimum Hepatocyte Density for Drug Activation.** The effect of hepatocyte density (24-hr exposure) upon growth stimulation and cyclophosphamide activation in A204 cell culture is shown in Chart 1. Hepatocyte coculture produced as much as 60% stimulation of tumor cell growth in the absence of cyclophosphamide at a hepatocyte:A204 cell ratio of 5:1. Inhibition of tumor cell growth in the presence of cyclophosphamide, 100 μg/ml, increased progressively with the number of hepatocytes with a maximum difference from control growth occurring at a hepatocyte:A204 cell ratio of 10:1. Spleen cells at similar density did not enhance the growth of A204 cells and produced only negligible cyclophosphamide activation. At a fixed hepatocyte:A204 cell ratio of 10:1, colony formation was inhibited 50% by 8 μg of cyclophosphamide/ml (Chart 2), a concentration achievable in patients.

**Viability and Functionality of Cultured Hepatocytes.** The viability and functionality of hepatocytes were assessed at different time intervals during soft-agar culture incubation (Table 3). After 4-hr incubation, viability (trypan blue exclusion) had decreased by 46%, while functionality (7-ethoxycoumarin O-dealkylation) had decreased by 55%. Loss of functionality measured by the diminished capacity of hepatocytes to activate cyclophosphamide in culture was 21% after 4 hr and 64% after 8 hr.

**Effect of Inducers and Inhibitors on Hepatocyte Drug Activation.** In order to further validate hepatocyte coculture as a prodrug activating system for the SACFA, we studied the effect of an inducer of

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**Table 1**

<table>
<thead>
<tr>
<th>Hepatocyte:A204 cell ratio</th>
<th>A204 cells alone (% of control)</th>
<th>A204 cells + cyclophosphamide (% of control)</th>
</tr>
</thead>
<tbody>
<tr>
<td>0:1</td>
<td>100 ± 7</td>
<td>67 ± 1</td>
</tr>
<tr>
<td>10:1</td>
<td>83 ± 13</td>
<td>15 ± 2</td>
</tr>
<tr>
<td>100:1</td>
<td>67 ± 10</td>
<td>0.1 ± 0.3</td>
</tr>
<tr>
<td>1000:1</td>
<td>33 ± 5</td>
<td>0.1 ± 0.3</td>
</tr>
</tbody>
</table>

* p < 0.05 compared to control cultures lacking cyclophosphamide.

**Table 2**

<table>
<thead>
<tr>
<th>Density and duration of hepatocyte coculture</th>
<th>A204 cells alone (% of control)</th>
<th>A204 cells + cyclophosphamide (% of control)</th>
</tr>
</thead>
<tbody>
<tr>
<td>Hepatocyte:A204 cell ratio (1:1)</td>
<td></td>
<td></td>
</tr>
<tr>
<td>1 hr</td>
<td>107.6 ± 6.3</td>
<td>107.0 ± 5.4</td>
</tr>
<tr>
<td>1 day</td>
<td>182.4 ± 21.5</td>
<td>85.9 ± 7.0</td>
</tr>
<tr>
<td>7 days</td>
<td>127.0 ± 11.3</td>
<td>97.2 ± 7.0</td>
</tr>
</tbody>
</table>

| Hepatocyte:A204 cell ratio (10:1)            |                                 |                                            |
| 1 hr                                        | 103.6 ± 4.4                     | 92.6 ± 5.1                                  |
| 1 day                                       | 208.3 ± 14.3                    | 3.4 ± 0.8                                   |
| 7 days                                      | 12.2 ± 4.1                      | 1.0 ± 0.4                                   |

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* p < 0.05 compared to control cultures lacking hepatocytes.

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**Chart 1.** Effect of hepatocyte (O, •) and spleen (D, •) cell number upon A204 cell colony formation. Open symbols in the absence of, and closed symbols in the presence of, 100 μg cyclophosphamide/ml. At this concentration, cyclophosphamide had no significant inhibitory effect upon growth. Duration of hepatocyte and spleen cell coculture was 24 hr. Percent survival is expressed relative to cultures lacking hepatocytes. Points, mean of 3 determinations; bars, S.E.

**Chart 2.** Efficiency of prodrug activation. A204 cells (10⁴) were cocultured with 10⁶ hepatocytes in the presence of cyclophosphamide for 24 hr. In the absence of hepatocytes, cyclophosphamide imposed no inhibition of colony formation by A204 cells. Points, mean of 3 determinations.
hepatic cytochrome P-450 (54), and pretreatment with carbon tetrachloride, a hepatotoxin which destroys more than 90% of cytochrome P-450 (51), upon the activation of cyclophosphamide by hepatocytes. Cyclophosphamide is known to require metabolic activation by cytochrome P-450 (13). These in vivo treatments had no effect upon the viability of hepatocytes isolated from the liver but had the predicted effects upon the extent of 7-ethoxy-9-coumarin O-dealkylation, a cytochrome P-450-dependent reaction. There were increases in 7-ethoxy-9-coumarin O-dealkylation by hepatocytes isolated from phenobarbital-induced rats and decreases in dealkylation by hepatocytes from carbon tetrachloride-pretreated rats (Table 4). The effect of phenobarbital and carbon tetrachloride pretreatments upon the cytotoxic activation of cyclophosphamide by hepatocytes is shown in Chart 3. In vivo phenobarbital pretreatment resulted in a marked increase in the in vitro hepatocyte activation of cyclophosphamide. For example, A204 cell colony formation in cultures containing cyclophosphamide and a nontreated hepatocyte:A204 cell ratio of 5:1 was 26% that of control, whereas colony formation in cultures containing the same density of phenobarbital-induced hepatocytes was 8% that of control. In contrast, cultures containing drug and the same density of hepatocytes prepared from carbon tetrachloride-pretreated rats exhibited colony formation 103% that of control. None of the in vivo pretreatments appeared to alter subsequent in vitro growth enhancement of A204 cells (Chart 4).

Effect of Hepatocyte Activation of Cyclophosphamide upon Colony Formation by a Human Lung Carcinoma Cell Line. The effect of cyclophosphamide activation upon colony formation by

Table 3

<table>
<thead>
<tr>
<th>Time (hr)</th>
<th>Trypan blue exclusion (%)</th>
<th>7-Ethoxy-9-coumarin O-dealkylation (pmol/min/10⁶ cells)</th>
<th>Cytotoxicity SACFA (% of control)</th>
</tr>
</thead>
<tbody>
<tr>
<td>0</td>
<td>77.6 ± 2.3a</td>
<td>40 ± 0</td>
<td>100</td>
</tr>
<tr>
<td>1</td>
<td>51.0 ± 8.1b</td>
<td>21 ± 5</td>
<td>106.2 ± 14.1</td>
</tr>
<tr>
<td>2</td>
<td>35.0 ± 9.1b</td>
<td>13 ± 3</td>
<td>93.3 ± 12.0</td>
</tr>
<tr>
<td>4</td>
<td>24.0 ± 9.1b</td>
<td>2 ± 1</td>
<td>78.6 ± 18.5</td>
</tr>
<tr>
<td>8</td>
<td>19.0 ± 6.6c</td>
<td>18 ± 2</td>
<td>36.0 ± 23.0</td>
</tr>
<tr>
<td>24</td>
<td>13.0 ± 6.6c</td>
<td>2 ± 1</td>
<td></td>
</tr>
</tbody>
</table>

a Mean ± S.E. of 3 separate experiments.  
b p < 0.05 compared to control.

Table 4

<table>
<thead>
<tr>
<th>Treatment</th>
<th>Trypan blue exclusion (% viable)</th>
<th>7-Ethoxy-9-coumarin O-dealkylation (pmol/min/10⁶ cells)</th>
</tr>
</thead>
<tbody>
<tr>
<td>Control</td>
<td>82 ± 2.6a</td>
<td>8</td>
</tr>
<tr>
<td>Phenobarbital</td>
<td>81 ± 2.5</td>
<td>4</td>
</tr>
<tr>
<td>Carbon tetrachloride</td>
<td>79 ± 3.1</td>
<td>2</td>
</tr>
</tbody>
</table>

a Mean ± S.E. of 3 separate experiments involving different animals.

A549 cells, a human lung carcinoma cell line, is shown in Table 5. A549 cells were slightly more resistant to the antiproliferative effect of hepatocyte-activated cyclophosphamide than were A204 cells. There was no apparent stimulation of A549 tumor cell growth by hepatocytes from control or carbon tetrachloride-pretreated animals over the range of hepatocyte densities used for drug activation.

Activation and Inactivation of Other Anticancer Drugs by Cultured Hepatocytes. The ability of isolated hepatocytes to alter the cytotoxicity of a variety of established and investigational anticancer drugs towards A204 cells is summarized in Table 6. Drugs can be placed into one of 3 categories on the basis of hepatocyte:tumor cell coculture results. Drugs showing cytotoxic activation on coculture with hepatocytes include cyclophosphamide, procarbazine, and indinom N-oxide; drugs which are inactivated by hepatocyte coculture include AZQ, BCNU, DTIC, 5-FU, fluorouracil, and MeCCNU; and drugs which are not affected by hepatocyte coculture include actinomycin D, CCNU, hexamethylmelamine, mitomycin C, 6-mercaptopurine, N-methylcytosine, and tricyclic nucleoside. Of interest is the fact that
DTIC, a light-sensitive drug (27), was more toxic to tumor cells in the SACFA following exposure of cultures with drug to light (results not shown). However, coculture with hepatocytes consistently resulted in inactivation of DTIC in the presence and absence of light.

**Cryopreservation.** Attempts were made to cryopreserve rat isolated hepatocytes using 2 methodologies. Manual freezing resulted in a marked loss of hepatocyte viability and functionality measured by trypan blue exclusion and 7-ethoxycoumarin dealkylation (Table 7). Automated freezing using a microcomputer-controlled freezer resulted in a mean hepatocyte viability of 37% and an O-dealkylation rate 39% that of control (nonfrozen) hepatocytes. However, use of cryopreserved hepatocytes in the SACFA resulted in only minimal cyclophosphamide activation (less than 10% that of freshly isolated hepatocytes). Various other procedures, such as varying the rate of freezing and use of the antioxidant α-tocopherol in the medium, failed to improve cell viability above that observed for automatic freezing. An additional problem encountered during cryopreservation experiments was that only about 55% of cells frozen were recovered as intact cells, and many were lysed presumably during freezing, storage, and/or thawing (no correction was made for the loss of cells when expressing viability). Although the drug activation capacity of hepatocytes in the SACFA was not maintained after cryopreservation, such cell preparations still possessed some capacity to enhance A204 cell proliferation (Table 7).

**DISCUSSION**

Isolated hepatocytes have been used as an activating system for in vitro toxicity studies with various types of target cells but not with human tumor cells in the SACFA (for a review, see Ref. 20). Isolated hepatocytes as an activating system for the SACFA offer several advantages over fractionated liver components. In addition to cytochrome P-450, hepatocytes possess a range of enzymes capable of activating prodrugs. There is increasing evidence in the literature that many drugs are activated to toxic metabolites by enzymes other than cytochrome P-450 (5, 8, 14, 31, 44, 48). In addition, hepatocytes might provide "barriers" to the diffusion and transport of drugs and reactive metabolites into and out of liver cells similar to those which may be encountered in vivo. It is conceivable that in vitro subcellular activating systems release short-lived reactive metabolites in close proximity to the target cell, resulting in cytotoxicity profiles not possible in vivo. Hepatocytes in fluid medium are compatible with tumor

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**Table 5**

<table>
<thead>
<tr>
<th>Cell Ratio</th>
<th>Nontreated</th>
<th>Carbon Tetrachloride Pretreated</th>
</tr>
</thead>
<tbody>
<tr>
<td></td>
<td>Hepatocytes: A549 cells alone (% of control)</td>
<td>Hepatocytes: A549 cells + cyclophosphamide (% of control)</td>
</tr>
<tr>
<td>2.5:1</td>
<td>94 ± 1%</td>
<td>108 ± 2%</td>
</tr>
<tr>
<td>5:1</td>
<td>100 ± 1%</td>
<td>92 ± 4%</td>
</tr>
<tr>
<td>10:1</td>
<td>98 ± 4%</td>
<td>57 ± 4%</td>
</tr>
<tr>
<td>20:1</td>
<td>87 ± 4%</td>
<td>25 ± 3%</td>
</tr>
</tbody>
</table>

* Mean ± S.E. of 3 determinations.
* *p < 0.05 compared to appropriate control cultures.
* *p < 0.01 compared to appropriate control cultures.

**Table 6**

<table>
<thead>
<tr>
<th>Drug</th>
<th>Without hepatocytes (μg/ml)</th>
<th>With hepatocytes (μg/ml)</th>
<th>Effect</th>
</tr>
</thead>
<tbody>
<tr>
<td>Cyclophosphamide</td>
<td>&gt;500</td>
<td>30.6 ± 1.0</td>
<td>Activated</td>
</tr>
<tr>
<td>Procarbazine</td>
<td>1614 ± 751</td>
<td>343 ± 505*</td>
<td>Activated</td>
</tr>
<tr>
<td>Indicine N-oxide</td>
<td>239 ± 92</td>
<td>171 ± 47*</td>
<td>Activated</td>
</tr>
<tr>
<td>Actinomycin D</td>
<td>0.02 ± 0.00</td>
<td>0.02 ± 0.00</td>
<td>No significant effect</td>
</tr>
<tr>
<td>CCNU</td>
<td>130 ± 49</td>
<td>102 ± 0.00</td>
<td>No significant effect</td>
</tr>
<tr>
<td>HMMA</td>
<td>&gt;100</td>
<td>&gt;100</td>
<td>No significant effect</td>
</tr>
<tr>
<td>Mitomycin C</td>
<td>0.01 ± 0.00</td>
<td>0.01 ± 0.00</td>
<td>No significant effect</td>
</tr>
<tr>
<td>6-MP</td>
<td>30.0 ± 1.3</td>
<td>22.2 ± 1.2</td>
<td>No significant effect</td>
</tr>
<tr>
<td>NMF</td>
<td>&gt;1000</td>
<td>&gt;1000</td>
<td>No significant effect</td>
</tr>
<tr>
<td>TGN</td>
<td>2.7 ± 0.1</td>
<td>4.0 ± 0.1</td>
<td>No significant effect</td>
</tr>
<tr>
<td>AZO</td>
<td>0.16 ± 0.00</td>
<td>0.47 ± 0.00</td>
<td>Inactivated</td>
</tr>
<tr>
<td>BCNU</td>
<td>13.2 ± 0.4</td>
<td>51.5 ± 4.0</td>
<td>Inactivated</td>
</tr>
<tr>
<td>DTIC</td>
<td>29.3 ± 2.4</td>
<td>44.0 ± 1.8</td>
<td>Inactivated</td>
</tr>
<tr>
<td>5-FU</td>
<td>4.5 ± 0.0</td>
<td>8.1 ± 0.1</td>
<td>Inactivated</td>
</tr>
<tr>
<td>Flotafur</td>
<td>40.9 ± 2.8</td>
<td>67.9 ± 10.1</td>
<td>Inactivated</td>
</tr>
<tr>
<td>MeCCNU</td>
<td>29.4 ± 0.6</td>
<td>47.9 ± 24.1</td>
<td>Inactivated</td>
</tr>
<tr>
<td>Vincaistine</td>
<td>2.5 ± 0.0</td>
<td>3.2 ± 0.0</td>
<td>Inactivated</td>
</tr>
</tbody>
</table>

* Mean ± S.E.
* *p < 0.01 compared to appropriate control without hepatocytes.
* *p < 0.05

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cells in soft-agarose cultures for longer durations than are possible with subcellular fractions.

In the present study, optimal hepatocyte density and coculture duration were determined through use of the prodrug, cyclophosphamide. Cyclophosphamide requires metabolic activation by the cytochrome P-450 of the mixed-function oxidase complex within the hepatic endoplasmic reticulum (13), and cytotoxic metabolites are released by isolated hepatocytes incubated with cyclophosphamide (6). Cyclophosphamide metabolites produced by the mixed-function oxidase also undergo further metabolism by other hepatic enzyme systems (6). It is noteworthy that some cyclophosphamide metabolites produced by hepatocytes cannot be detected following incubation with subcellular fractions (6). At concentrations up to 100 μg/ml, cyclophosphamide alone had no significant inhibitory effect upon tumor cell proliferation. In the presence of an optimal hepatocyte density, colony formation by A204 cells was inhibited 50% by 8 μg of cyclophosphamide/ml, which is below peak concentrations of cyclophosphamide achieved therapeutically in human plasma (28). Hepatocytes and drug were applied to tumor cell cultures for a period of 24 hr. Such a duration appears to permit a more efficient degree of in vitro drug metabolism than shorter incubations. Although viability and functionality indices are much reduced at 24 hr (Table 3), hepatocyte metabolism between the 8- and 24-hr time intervals contributes significantly to the total quantity of drug liberated into culture. Although such a duration is longer than therapeutic cyclophosphamide concentrations are maintained by most clinical regimens (9, 25), it may not be possible to devise an in vitro system consistent with all aspects of in vivo drug exposure. A 24-hr incubation appears to be an appropriate and operationally convenient duration for hepatocyte:tumor cell coculture and adaptable to chemosensitivity testing in the SACFA (55).

There have been reports that cryopreservation of rat isolated hepatocytes (32, 33, 45) does not alter levels of cytochrome P-450 and other microsomal enzymes, although there is loss of some specific cell functions, such as gluconeogenesis (33). Our attempts to utilize cryopreserved hepatocytes for the purpose of subsequent in vitro drug activation were unsuccessful. Even under optimal conditions for freezing and thawing, cells exhibited only about 10% functionality (measured in terms of cyclophosphamide activation in the SACFA). Linear rates of cooling, 1–7°C/min (18, 33, 45), provided by a microcomputer-controlled freezer were observed to substantially improve cell viability over that of manual freezing but did not yield hepatocytes capable of adequate metabolic activation (although a persistence of growth-enhancing properties was noted). Other variables known to affect viability of frozen cells, such as storage temperature (32), rate of thawing (18), and postthaw handling (58), did not appreciably affect hepatocyte drug activation.

Several examples of activation, inactivation, and no alteration in cytotoxicity were observed for a variety of anticancer drugs incubated in hepatocyte:tumor cell coculture. Procarbazine, known to be converted in high yield to potentially cytotoxic metabolites by cytochrome P-450 (61), exhibited marked activity in hepatocyte:tumor cell coculture. Another drug activated by hepatocytes was indocine N-oxide, an investigational anticancer agent with activity against human leukemia (34). While the mechanism of action of indocine N-oxide is not known (46), it is readily metabolized by rabbit liver preparations (47) and can be hepatotoxic to humans in high dosage (34). Whether in vivo hepatotoxicity results from the generation of toxic metabolites within liver cells is not known.

The cytotoxicity of some drugs was unaffected when cultured with hepatocytes. These drugs include actinomycin D, a drug which binds to DNA without the need for metabolic interaction and which probably undergoes little metabolism in humans (24). The cytotoxicity of mitomycin C is thought to result from activation within tumor cells to a reactive species that alkylates and cross-links DNA. Although mitomycin C can be metabolized by hepatocytes in the SACFA in air. 6-Mercaptopurine undergoes extensive biotransformation in vivo and is oxidized by xanthine oxidase (37) as well as by other hepatic enzymes (30). However, in the present study, cytotoxicity of 6-mercaptopurine was not affected by hepatocyte coculture. Likewise, tricyclic nucleoside, an investigational drug, exhibited unaltered toxicity in the system. While conflicting claims exist concerning the ability of hepatic preparations to metabolize N-methylformamide (4, 23), the results of the present study, where no cytotoxicity was seen with or without hepatocytes at drug concentrations below 1 mg/ml, suggest that the liver is not a major site for metabolism of this agent. The nitrosoureas, CCNU and MeCCNU, are hydroxylated (38) to a series of metabolites with antitumor properties similar to those of the parent compound (62). Cytotoxicity of CCNU showed no change on coinubcation with hepatocytes, although MeCCNU exhibited some loss of activity.

An unexpected finding of the present study was the absence of hexamethylmelamine activation by hepatocytes. Although the mechanism of action of hexamethylmelamine is unknown, the

<table>
<thead>
<tr>
<th>Trypan blue exclusion (%)</th>
<th>7-Ethoxycoumarin deethylase (pmol/min/10^6 cells)</th>
<th>Cyclophosphamide activation (ID₅₀)</th>
<th>Growth enhancement (%)</th>
</tr>
</thead>
<tbody>
<tr>
<td>Control</td>
<td>77.0 ± 1.8 (17)</td>
<td>52.9 ± 6.8 (17)</td>
<td>5 ± 0 (3)</td>
</tr>
<tr>
<td>Manual freezing</td>
<td>12.0 ± 2.2 (12)</td>
<td>6.5 ± 3.2 (12)</td>
<td>20.6 ± 2.8 (17)</td>
</tr>
<tr>
<td>Automatic freezing</td>
<td>28.4 ± 3.0 (17)</td>
<td></td>
<td></td>
</tr>
</tbody>
</table>

*ID₅₀, hepatocyte density expressed as hepatocyte:A204 cell ratio producing 50% inhibition of tumor cell growth in the presence of cyclophosphamide (100 μg/ml).

*Growth enhancement is expressed as maximum enhancement relative to A204 cell culture lacking hepatocytes.

*Mean ± S.E.

*Numbers in parentheses, number of determinations.
Drug Metabolism in Hepatocyte:Tumor Cell Coculture

drug is extensively metabolized by hepatic microsomal cytochrome P-450 to demethylated products (50) and other intermediates which bind covalently to microsomal macromolecules (2). It has been reported that hexamethylmelamine can be activated to cytotoxic intermediates when tumor cells are incubated for periods of up to 2 hr with cofactor-fortified hepatic postmitochondrial supernatant (42, 50). There is also a report that hexamethylmelamine cytotoxicity can be observed in vitro in the absence of a hepatic postmitochondrial activating system (15). In the present study, A204 cell proliferation was not altered by exposure to high concentrations of hexamethylmelamine for 24 hr in either the absence or presence of hepatocytes. Since this same tumor cell line was used to demonstrate the increased cytotoxicity of hexamethylmelamine when incubated with the hepatic postmitochondrial fraction (29), it is probable that cytotoxic intermediates, if formed by metabolism of hexamethylmelamine within intact hepatocytes, are further metabolized or unable to pass through the hepatocyte membrane in sufficient amounts to achieve cytotoxic extracellular concentrations.

Drugs which are inactivated by coculture with hepatocytes include BCNU, DTIC, AZQ, 5-FU, ftorafur, MeCCNU, and vincristine. BCNU has been reported to be enzymatically denitrosated by hepatic microsomal enzymes (27), resulting in loss of antitumor activity (35). While DTIC is believed to require microsomal demethylation to yield an active alkylating species (53), evidence that one specific metabolite is responsible for cell death is lacking (29). Although Metelmann and Von Hoff (40) have reported cytotoxic activation of DTIC using a commercial preparation of rat hepatic postmitochondrial supernatant, the results are unusual in that a NADPH-generating system was not necessary for the activation of DTIC. It is not clear whether their observed end point resulted from true enzymatic activation of DTIC or nonenzymatic activation, perhaps by light during the preincubation (22). In addition, the authors found it necessary to preincubate drug with the postmitochondrial fraction for 1 hr and then to remove the sedimentable portion of the fraction by low-speed centrifugation to avoid direct inhibition of tumor cell growth by the postmitochondrial fraction. In the present study, DTIC was consistently inactivated by hepatocytes in the SACFA, irrespective of whether the cells were exposed to light or incubated in the dark. Although AZQ is metabolized by mouse liver homogenate to several metabolites, it is not known whether any of these metabolites possess antitumor activity (56). The results of the present study suggest that AZQ is converted to inactive metabolites by hepatocyte metabolism. 5-FU is well known to be extensively metabolized by liver to inactive products (43) and not surprisingly showed less activity when cocultured with hepatocytes. Ftorafur, which is metabolized to 5-FU by the liver (3), also showed significant loss of cytotoxicity when cocultured with hepatocytes. Whether conversion of ftorafur to 5-FU within target cells is more important than conversion to circulating 5-FU for activity has not been resolved (11). Although vincristine has been reported not to be metabolized to a large extent by rat liver (10), in the present study, vincristine showed significantly less activity when cocultured with hepatocytes.

In summary, rat hepatocytes in fluid medium were observed to be compatible with soft-agarose tumor cell culture and to provide an efficient means to activate as well as inactivate a number of cancer chemotherapeutic agents, while some agents were unaffected. Although cryopreserved hepatocytes lack adequate drug metabolism activity, freshly isolated hepatocytes in coculture with human tumor cell lines appear to be a useful system for identifying cancer chemotherapeutic agents which may require or are susceptible to hepatic metabolism.

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Activation and Inactivation of Cancer Chemotherapeutic Agents by Rat Hepatocytes Cocultured with Human Tumor Cell Lines


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