A Novel Dye Exclusion Method for Testing in Vitro Chemosensitivity of Human Tumors

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ABSTRACT

Dissociated cancer cells are exposed to antineoplastic drugs (5 x 10^6 viable cells/drug for 1 hr or continuously) and cultured for 4 to 6 days in liquid medium. Cells are then stained with Fast green dye, sedimented onto slides with a Cytospin centrifuge, and counterstained with a modified hematoxylin and eosin technique. Dead cells stain with Fast green, and living cells stain with hematoxylin and eosin. Cell kill is calculated as percentage of control based on the relative numbers of living tumor cells, living non-tumor cells, and dead cells. Drug sensitivity could be assayed in 125 of 162 specimens of human neoplasms obtained from malignant effusions (16 of 18), excisional biopsies (31 of 44), needle biopsies (34 of 47), endoscopic biopsies (18 of 23), peripheral blood samples (19 of 20), and bone marrow aspirates (five of seven). The assays were successful (median of ten drugs tested) in: 46 of 64 adenocarcinomas; four of 11 squamous cell carcinomas; five of seven lymphomas; six of seven melanomas; two of four sarcomas; 18 of 20 transitional-cell carcinomas; 14 of 15 small-cell carcinomas; seven of eight myelomas; 12 of 12 chronic lymphocytic leukemias; seven of nine acute leukemias, and four of five “undifferentiated” carcinomas. The assay results demonstrated a strong correlation between the in vitro chemosensitivity of different types of tumors and the known clinical response patterns of these tumors. This assay can be used to determine which specific cells are killed in a heterogeneous cell population. Further work is needed to determine if this assay may be useful in blind screening trials for antineoplastic agents or if it may be of clinical use in predicting response to agents which are not cycle specific.

INTRODUCTION

The perfection of a practical test to evaluate the relative efficacies of different antineoplastic drugs in the treatment of human tumors prior to the initiation of clinical chemotherapy has been an elusive goal since the pioneering efforts of Black and Speer (5) in the early 1950s. Many authors have reported positive correlations between the results of in vitro assays and the results of clinical chemotherapy in humans (2, 4, 5, 7-10, 13, 15, 17-21, 24, 26, 27, 29, 30, 32, 33). In spite of this, a role for the use of in vitro assays of chemosensitivity in clinical oncology has not yet been established. Recently, Salmon et al. (26, 27) have adapted an agar cloning assay for use in determining the in vitro chemosensitivity of human tumors. Although promising in terms of its predictive accuracy, this assay has technical disadvantages limiting its application in clinical oncology and preclinical drug screening.

The ability of a viable cell to exclude a variety of dyes in the presence of a physiological salt solution has long been used as a basis for quantifying cell death in vitro (10, 14, 23, 28). Durkin et al. (10) reported a perfect correlation between the results of an in vitro assay based on dye exclusion and the results of chemotherapy in the treatment of 7 patients with non-Hodgkin’s lymphomas. Dye exclusion assays are customarily performed by counting wet preparations of cells in a hemocytometer in the presence of a dye such as trypan blue, eosin, erythrosin-B, or nigrosin (10, 14, 23, 28). Among the disadvantages of this technique are (a) the necessity of evaluating the wet preparation within a crucial time after the introduction of the dye, since the “viable” cells will begin to take up dye after prolonged exposure; (b) a poor ability to distinguish between viable tumor cells and viable non-tumor cells present in the preparation, since the viable cells remain unstained and difficult to identify; and (c) the possibility of seriously underestimating cell kill (3, 25, 34, 35).

This paper will describe a new dye-staining technique designed to overcome the first 2 of the above disadvantages. The third disadvantage may be potentially overcome by using in vitro drug concentrations which are appropriate for dye exclusion assays rather than those which are appropriate for clonogenic assays.

MATERIALS AND METHODS

Drugs. VP-16 was prepared by diluting fresh surplus stock solutions, obtained from the oncology outpatient clinic, with 0.15 M NaCl. All other drugs were obtained in powder form from the Division of Cancer Treatment, the National Cancer Institute, Bethesda, Md. All drugs were diluted to a concentration equal to 10 times the desired final concentration in 0.15 M NaCl and stored at -70°C until use. Nitrogen mustard was stable for 3 months when stored in this manner, as monitored by induced cytotoxicity, Cancer Res., in press, 1983.

Established Cell Line. HL-60 human promyelocytic leukemia cells were maintained in continuous suspension culture in Roswell Park Memorial Institute Tissue Culture Medium 1640 with 15% fetal calf serum (6). The doubling time of these cells was 30 to 36 hr.

Tumor Cell Preparation. Fresh human solid tumor specimens were placed in Roswell Park Memorial Institute Tissue Culture Medium 1640 with 10% heat-inactivated fetal bovine serum and penicillin (100 units/ml); streptomycin (100 μg/ml) for transport to the laboratory. Peripheral blood and marrow samples were heparinized. Malignant effusions were kept at room temperature before being assayed. Solid tissue samples were dissociated by teasing them apart with 25-gauge needles after grossly mincing the specimen with scalpels and scissors. In some experiments, minced tissue was exposed for 2 hr to 0.8% collagenase


2 Supported by the United States Veterans Administration.

3 To whom requests for reprints should be addressed.

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I and 0.002% DNase I (22). Excessive trauma was avoided, since it
was not necessary to obtain a perfect single-cell suspension for the
purposes of the assay to be described. Cells were collected from
effusions by means of centrifugation. Lymphocytes were obtained from
leukemic blood by expelling the leukocyte-rich plasma which resulted
by allowing the RBCs to settle after tapping the phlebotomy syringe to
the wall in a vertical position. Bloody specimens or specimens with a
trypan blue viability <40% were centrifuged over lymphocyte separat-
ning medium (Litton Bionetics, Kensington, Md.), and then cells at the
interface were collected with a Pasteur pipet. The number of viable
nucleated cells was quantified in the standard fashion using 0.4% trypan blue in 0.15 M NaCl and a hemocytometer counting chamber.

**Drug Sensitivity Assay.** The desired number of viable cells was then
 aliquoted into individual polypropylene tubes. Polypropylene was used
to discourage cell attachment to the wall of the tube (11).

For each drug to be assayed, 50,000 to 500,000 viable cells were
required. In cases where meager cell numbers were obtained, a minimum of 50,000 cells/assay tube was used. In cases where meager cell
numbers were obtained, a minimum of 50,000 cells/assay tube was used.
These cells were suspended in 0.9 ml of the complete medium
described above, and 0.1 ml of the 10x-concentrated drug solution
(or 0.15 M NaCl as a control) was added to begin the assay. The drug
concentrations were chosen on the basis of preliminary studies in
established cell lines. The final drug concentrations in the cell incu-
bation mixture were as follows: doxorubicin, 0.4 to 1.2 μg/ml; nitrogen
mustard, 0.7 to 3.5 μg/ml; melphalan, 2.5 to 12 μg/ml; VP-16, 125
μg/ml; SFU, 10 to 25 μg/ml; and dexamethasone, 0.4 μg/ml.

After the 60-min incubation at 37°C, 3 ml of fresh complete medium
were added, and the cells were centrifuged, washed twice with fresh
medium, and resuspended in 1.0 ml of fresh medium for short-term
culture at 37°C under a humidified atmosphere of 94% air: 6% CO₂. In
some experiments, the drugs were not washed out but remained for
the entire 4-day period of short-term culture.

**Assessment of In Vitro Cell Kill.** In specimens containing 500,000
cells/ml, the following assay procedure was used: 1.0 ml of a solution of
2% Fast green (Matheson Co., East Rutherford, N. J.) in 0.15 M
NaCl was added to each of the tubes which were then agitated on a
vortex mixer. After precisely 10 min following addition of the dye, the
solution was vigorously pipetted up and down with a Pasteur pipet, and
8 drops of the cell suspension (again agitated) were added to the chamber of a Cytospin centrifuge (Shandon Southern Instruments, Inc.,
Sewickley, Pa.). The cell suspension was centrifuged at 1200 rpm for
7 min, and the filter papers were carefully removed to avoid disturbing
the cell button which was deposited on the slide (12, 16). The resulting
slide contained green-stained cells (dead), unstained clear cells (living),
and possible RBCs. This slide was then counterstained with a modified
H&E stain.

Up to this point, the cells were not treated with a fixative. Treatment
with alcohol resulted in a loss of green stain from the dead cells, and
possible RBCs. This slide was then counterstained with a modified
H&E stain.

All values were then expressed as a percentage of control by multiply-
ing each number by 100 and then dividing by the value of the percent-
age of living tumor cells expression for the control (0.15 M NaCl without
drugs) assay slide. The percentage of control value so obtained is
referred to as percentage of surviving tumor cells.

In experiments in which the number of viable tumor cells was
substantially exceeded by the sum of the number of dead cells plus the
number of living non-tumor cells, the cells were counted by using a special
eyepiece objective (Whipple disc, Bausch and Lomb No. 31-16-69;
WVR Scientific, Norwalk, Calif.). This objective projected onto the
microscope field a large square which was subdivided into 100 smaller
squares. One of the smaller squares was, in turn, subdivided into 25
smaller squares. The number of living tumor cells was, for example,
equal to only one-tenth of the number of living non-tumor cells and
dead cells, then living tumor cells would be counted if they appeared
in the largest square of the grid, while living non-tumor and dead cells
would only be counted if they appeared in 10 preselected smaller
squares. In the data calculations, the numbers of living non-tumor and
dead cells would be first multiplied by 10, and the resulting corrected
numbers would be used in the above equation to calculate the per-
centage of living tumor cells. The purpose of using the grid in this
manner was to: (a) ensure that adequate numbers of tumor cells were
counted to give a reliable result; and (b) minimize the tedium involved
in counting excessive numbers of dead and non-tumor cells. In general,
a total of 300 cells (living tumor cells plus living non-tumor cells plus
dead cells) was counted for each slide from randomly chosen fields of
different areas of the slide. Counting 100 cells on each of 3 triplicate
slides was placed in eosin solution [% eosin (100 ml); 1% phloxine (10 ml); 95% ethanol (780 ml); glacial acetic acid (4 ml)] for
precisely 30 sec, during which time fixation took place. The slides
were then briefly dehydrated by 2 quick dips, successively, in 95% alcohol, 95% alcohol, 100% alcohol, 100% alcohol, xyline, and xy-
ylene. The slides were then coverslipped using mounting balsam. The
resulting slides could be stored for examination for more than 15
months without losing their staining characteristics. Upon examination,
the dead cells remained stained a brilliant green (which appears more
bluish in the photomicrographs, due to the camera filters), and the
living cells had their characteristic appearance with H&E (Figs. 1 to 4).
Preparations which originally had heavy RBC contamination prior to
lysis demonstrated a "reticular-nodular" background representing
debris from the process of lysis. The morphology of the dead cells ranged
from the discrete, well-preserved cell architecture (Fig. 1) to the diffuse,
smudged appearance of severely degenerated collections of cells (Fig.
2).

It is important to note that the Fast green dye solution was filtered
daily, and the slides were cleaned with ethanol before use. These
precautions minimized the amount of debris concentrated within the
cell button. In experiments in which meager cell numbers were obtained, only
50,000 to 100,000 viable nucleated cells were assayed in each tube.
A final incubation volume of 1.0 ml was again used. However, at the
end of the 4-day period of incubation, the specimens were centrifuged,
and all but 0.2 ml of the supernatant was removed. Two-tenths ml of
Fast green solution was then added, and the entire mixture (0.4 ml or
8 drops) was added to the cytocentrifuge chamber after 10 min.
For interpretation of the assays, the slides were examined micro-
scopically. In experiments in which the number of living tumor cells
(stained with H&E, not Fast green) was approximately equal to or
greater than the sum of the number of dead cells (stained with Fast
green) plus the number of living non-tumor cells, the slides were
counted by using the ehrler ratio of eell and rcalculating
\[
\% \text{ of living tumor cells} = \frac{\text{no. of living tumor cells}}{\text{no. of living tumor cells} + \text{no. of dead cells} + \text{no. of living non-tumor cells}} \times 100
\]
All values were then expressed as a percentage of control by multiply-
ing each number by 100 and then dividing by the value of the percent-
age of living tumor cells expression for the control (0.15 M NaCl without
drugs) assay slide. The percentage of control value so obtained is
referred to as percentage of surviving tumor cells.

In experiments in which the number of viable tumor cells was
substantially exceeded by the sum of the number of dead cells plus the
number of living non-tumor cells, the cells were counted with a special

slides was not meaningfully different from counting 300 cells on a single slide.

Some tumor cell types (such as small-cell lung cancer and bladder cancer) in liquid culture tended to form clumps which were not easily dispersed. These specimens presented some problems in counting, since the clumps tended to sediment onto one specific quadrant of the cell “disc” after cytocentrifugation. This left some areas of the slide very hypercellular compared to other areas. Additionally, quantifying cell numbers accurately when the cells were in a large clump was difficult. In these cases, living tumor cells were counted in only one or several of the smaller squares in each high-power field, and an effort was made to sample fields randomly from all areas of the cell disc. By “sampling” small areas of a large number of high-power fields, it was possible to get reproducibly quantitative results from excessively clumpy slides. In later experiments, it was found that cells could be partially declumped by mixing them with an equal volume of 30 mM EDTA:10 mM dithiothreitol, dissolved in phosphate-buffered saline (pH 7.4).

RESULTS

Technical Factors Related to Staining. Fast green was chosen for use as a vital stain because of its tenacity in being retained by the dead cells during the counterstaining process and because its striking color could not be confused with normal H&E dye shadings. While no direct proof was obtained that Fast green specifically stained the dead cells, the estimates of cell viability obtained with traditional trypan blue wet preparations and with Fast green wet preparations were similar. Additionally, trypan blue could also be used to stain the dead cells in the dry slide technique described here, with similar estimates of cell viability being obtained compared to dry slides prepared with Fast green (data not shown). The dry slide preparations frequently made it possible to detect severely degenerated (“ghost”) cells which were not visible in the presence of dye in the wet preparations because of insufficient contrast. Counterstaining with Wright’s stain gave unsatisfactory results, because the cells stained previously with Fast green lost their distinctive green color during counterstaining. The morphology of the living cells was reasonably well preserved during the staining-counterstaining process (Figs. 1 to 4), but it was not perfect because of a lack of prefixation and the inevitable presence of some drying artifact.

In preparations from solid tumor samples, it was relatively easy to distinguish between tumor cells and non-tumor cells on the stained preparations. Because fibroblasts did not grow in the polypropylene tubes, the vast majority of the surviving non-tumor cells were either histiocytes or lymphocytes which could usually be distinguished from the living tumor cells with ease. It was also very easy to distinguish between lymphocytes and granulocytes on peripheral blood preparations and to distinguish plasma cells from other marrow elements in slides prepared after the short-term liquid culture of bone marrow from myeloma patients (Figs. 1 and 2). On the other hand, it was sometimes difficult for an observer not trained as a cytopathologist to distinguish between pleural fluid tumor cells and mesothelial cells in the dry slide preparations. Therefore, slides of pleural fluids were made prior to setting up drug assays to determine if tumor cells were identifiable and could be readily distinguished from the background cells. After 4 days in culture, tumor cells tended to stain more hyperchromatically than did mesothelial cells, which aided in their identification.
Table 1

<table>
<thead>
<tr>
<th>Specimen type</th>
<th>Assays successful/specimens received</th>
</tr>
</thead>
<tbody>
<tr>
<td>Effusions</td>
<td>16/18</td>
</tr>
<tr>
<td>Blood</td>
<td>19/20</td>
</tr>
<tr>
<td>Marrow</td>
<td>5/7</td>
</tr>
<tr>
<td>Open biopsy</td>
<td>31/44</td>
</tr>
<tr>
<td>Needle biopsy</td>
<td>34/47</td>
</tr>
<tr>
<td>Bronchoscopy</td>
<td>2/5</td>
</tr>
<tr>
<td>Cystoscopy</td>
<td>16/18</td>
</tr>
<tr>
<td>Skin punch</td>
<td>2/3</td>
</tr>
<tr>
<td>Total</td>
<td>125/162 (77%)</td>
</tr>
</tbody>
</table>

Histology

<table>
<thead>
<tr>
<th>Tumor Type</th>
<th>Assays successful/specimens received</th>
</tr>
</thead>
<tbody>
<tr>
<td>Adenocarcinoma</td>
<td>46/64</td>
</tr>
<tr>
<td>Squamous cell</td>
<td>4/11</td>
</tr>
<tr>
<td>Small cell</td>
<td>14/15</td>
</tr>
<tr>
<td>Myeloma</td>
<td>7/8</td>
</tr>
<tr>
<td>CLL</td>
<td>12/12</td>
</tr>
<tr>
<td>Lymphoma</td>
<td>5/7</td>
</tr>
<tr>
<td>Sarcoma</td>
<td>2/4</td>
</tr>
<tr>
<td>Bladder</td>
<td>18/20</td>
</tr>
<tr>
<td>Acute leukemia</td>
<td>7/9</td>
</tr>
<tr>
<td>Melanoma</td>
<td>6/7</td>
</tr>
<tr>
<td>&quot;Undifferentiated&quot;</td>
<td>4/5</td>
</tr>
<tr>
<td>Total</td>
<td>125/162 (77%)</td>
</tr>
</tbody>
</table>

actually tested a mean of 11 drugs and a median of 10 drugs per successful assay, considering only the solid tumor specimens. The potential number of drug assays on effusion and blood samples was almost never limited by the number of cells present.

Drugs Tested. Although we assayed a large spectrum of drugs, the drugs for which we have the largest numbers of clinical correlations are nitrogen mustard (0.7 and 3.5 μg/ml), melphalan (2.5 and 12.5 μg/ml), doxorubicin (1.2 μg/ml), VP-16 (125 μg/ml), dexamethasone (0.4 μg/ml), and 5-FU. The latter drug was not active in our assay system at concentrations up to 25 μg/ml with a 1-hr drug exposure. Accordingly, this drug was tested at concentrations ranging from 10 to 100 μg/ml using a continuous exposure (Chart 2). Based on these data, 25 μg/ml were chosen as the concentration at which 5-FU would be routinely tested using a continuous drug exposure. Under these conditions, solid tumors were heterogeneous in their sensitivity to 5-FU with approximately 20% of the solid tumor specimens demonstrating <30% cell survival (Chart 3). Dexamethasone was also tested with a continuous exposure and was "active" in 3 of 9 cases of CLL and multiple myeloma.

In the other 4 drugs, a 1-hr drug exposure was sufficient to produce <30% cell survival in about 20% of the solid tumor specimens. This is illustrated for doxorubicin in Chart 3. In addition to the single-agent assays, the following 3-drug combination was tested in 22 solid tumor specimens: nitrogen mustard (0.6 μg/ml) plus doxorubicin (0.2 μg/ml) for 1 hr, followed by 5-FU (10 μg/ml) continuous exposure. Three of 22 solid tumor specimens had <30% cell survival after treatment with this combination.

Relationship of Control Cell Viability to Chemosensitivity. The viability of the control tumor cells after 4 days in culture could be determined accurately only in cultures in which there were no appreciable numbers of non-tumor cells, since we did not determine whether the "dead" cells were originally tumor or non-tumor cells. The median viabilities for the control cells after 4 days in culture were as follows: adenocarcinomas, 33% (n = 20); small-cell carcinomas, 35% (n = 7); and transitional-cell carcinomas, 13% (n = 15). Absolute control viabilities for tumor cells from solid tumor specimens ranged from 0.4 to 93%. In solid tumors, there was no clear relationship between the control viability after 4 days in culture and the in vitro chemosensitivity to the drugs tested. However, in 2 cases of CLL, lymphocytes from peripheral blood samples had a control viability of >50% after 4 days in culture, while lymphocytes from synchronous needle lymph node biopsies had control viabilities of <5% after 4 days in culture. In both cases, the peripheral blood cells appeared to be substantially less sensitive to doxorubicin than were the lymph node cells. It is not known whether this represented an actual difference in chemosensitivity between 2 different cell populations or merely an in vitro artifact relating to extracorporeal cell viability.

In Vitro “Phase II Trials.” In order to determine if the assay was potentially useful for new drug screening, the in vitro sensitivity of the tumor specimens was examined by histological type (Table 2). The findings presented in Table 2 seemed quite compatible with clinical experience. In addition, dexamethasone (0.4 μg/ml, continuous exposure) was found to be "active" (<30% cell survival) in 7 of 13 specimens of lymphoid neoplasms (CLL, multiple myeloma, acute lymphoblastic leukemia, non-Hodgkin’s lymphoma). Dexamethasone was active in 0 of 14 specimens of nonlymphoid neoplasms (solid tumors and acute nonlymphocytic leukemia). The above findings strongly imply that the assay might be useful in targeting new drugs for clinical trials in selected neoplasms.

Clinical Correlations. Most patients were treated with drug combinations rather than with single agents. For this reason, it
was extremely difficult to make valid in vitro-in vivo correlations. Table 3 lists correlations which could be made from our first 162 attempted assays. For purposes of this analysis, the following drug concentrations were used: doxorubicin, 1.2 μg/ml; nitrogen mustard, 3.5 μg/ml (solid tumors) and 0.7 μg/ml (CLL and multiple myeloma); melphalan, 12.5 μg/ml (solid tumors) and 2.5 μg/ml (CLL and multiple myeloma); 5-FU, 25 μg/ml (continuous exposure); VP-16, 125 μg/ml; and dexamethasone, 0.4 μg/ml (continuous exposure). Lower concentrations of alkylating agents were used in the clinical correlations for CLL and myeloma because multiple drug concentrations were tested in these neoplasms, and the lower concentrations gave the best correlations. The lower concentrations were not generally tested in solid tumors. These data suggest that a degree of correlation exists between in vitro and in vivo sensitivity for individual patients, but the data are not adequate to allow for an estimate of "true positive" or "true negative" rates. Based upon our experience to date, we suggest, in Table 4, criteria for tumor sensitivity to selected drugs which may be tested in future prospective trials.

![Doxorubicin Chart](chart.png)

Chart 3. Tumor cell survival after a 1 hr exposure to doxorubicin (1.2 μg/ml) or a continuous exposure to 5-FU (25 μg/ml). Each symbol represents an individual assay performed on cells from a different patient. No patient is represented more than once, □, solid tumors; ○, hematological neoplasms. Lines at 30% arbitrary cutoffs between "sensitive" and "resistant" for the preliminary clinical correlations.

### DISCUSSION

The ability to predict the sensitivity of individual human tumors to the effects of available cytotoxic agents in advance of their actual use in vivo would have a major impact on the practice of clinical oncology. Likewise, the ability to screen in vitro for antitumor activity of experimental antitumor agents in freshly obtained human tumor specimens could potentially improve the yield in empirical drug screening (31). Among the many different assays proposed previously for use in the above ways (see references in "introduction"), the use of an agar cloning assay (26, 27) has attracted much attention recently. The National Cancer Institute has decided to mount a large-scale effort to incorporate this latter assay into its mammoth drug-screening program (NIH request for Proposal NCI-IM-07327, April 1, 1980).

When compared to the agar cloning assay, the assay described here may have certain unique advantages. This assay does not depend upon cell growth in culture, making it possible to assay neoplasms, such as CLL, which do not grow in agar. Additionally, the assay does not require complete cell dispersal into a single-cell suspension. In order to assay 11 drugs at a single concentration (plus one control), a minimum of 600,000 cells is required, which is only about one-tenth of the number of cells required for assaying a similar number of drugs using the agar cloning assay (without duplicate cultures). Fungal contamination is much less a problem because of the shorter time required in culture. This shorter period of time (4 days) may have obvious advantages in a clinical situation. In our own experience, the assay described herein, while not simple, is easier and less expensive to perform and interpret than the agar cloning assay. Permanent slides are made so that the results of the assay may be interpreted, rechecked, or compared to other assays at any convenient future time. In preparations from specimens with mixed cell populations, such as bone marrow specimens, it is possible to determine differences in drug effects on the different populations of cells within the

### Table 2

<table>
<thead>
<tr>
<th>Drug</th>
<th>Small-cell lung cancer</th>
<th>Non-small-cell lung cancer</th>
<th>Ovary</th>
<th>Breast</th>
<th>Multiple myeloma and CLL</th>
</tr>
</thead>
<tbody>
<tr>
<td>Doxorubicin (1.2 μg/ml; 1 hr)</td>
<td>10/14</td>
<td>1/29</td>
<td>4/25</td>
<td>3/10</td>
<td>9/20</td>
</tr>
<tr>
<td>VP-16 (1 25 μg/ml; 1 hr)</td>
<td>2/10</td>
<td>0/11</td>
<td>0/3</td>
<td>0/3</td>
<td>8/16</td>
</tr>
<tr>
<td>5-FU (25 μg/ml; continuous)</td>
<td>4/10</td>
<td>4/21&lt;sup&gt;a&lt;/sup&gt;</td>
<td>3/17</td>
<td>1/7</td>
<td>2/6</td>
</tr>
<tr>
<td>Nitrogen mustard (3.5 μg/ml; 1 hr)</td>
<td>8/15</td>
<td>1/25</td>
<td>5/21</td>
<td>3/8</td>
<td>13/19</td>
</tr>
<tr>
<td>L-Phenylalanine mustard (melphalan) (12.5 μg/ml; 1 hr)</td>
<td>2/9</td>
<td>0/17</td>
<td>3/16</td>
<td>2/6</td>
<td>13/16</td>
</tr>
</tbody>
</table>

<sup>a</sup> All non-small-cell lung cancer specimens sensitive to 5-FU were adenocarcinomas.
<table>
<thead>
<tr>
<th>Patient</th>
<th>Type of neoplasm</th>
<th>In vitro result</th>
<th>In vivo result</th>
</tr>
</thead>
<tbody>
<tr>
<td>1 Ovary</td>
<td>S HN2, S DOXO</td>
<td>S CYCLO + DOXO (90% disappearance of massive intrapelvic tumor confirmed by computed tomography scan and second-look laparotomy). After tumor relapse, 2 different assays were then showing in vitro R DOXO and HN2.</td>
<td></td>
</tr>
<tr>
<td>2 CLL</td>
<td>S HN2, S L-PAM</td>
<td>S Chlorambucil (WBC count went from 96,000 to 5,000 after 1 mo. of treatment. No recurrence in 6 mos. off treatment).</td>
<td></td>
</tr>
<tr>
<td>3 CLL</td>
<td>S L-PAM, S DEX</td>
<td>S L-PAM + DEX (80% decrease in massive lymphadenopathy for 2 mos. before disease progression. Reassay then showed in vitro R L-PAM. DEX not reasayed.)</td>
<td>Mixed-response DOXO (disappearance of lesion biopsied with needle, disappearance of largest pulmonary tumor nodule. Stabilization of remaining lesions for 6 wk., followed by disease progression. Biopsy of lesions at 2 different sites of disease progression both then showed in vitro R DOXO).</td>
</tr>
<tr>
<td>4 Small-cell lung</td>
<td>S DOXO</td>
<td>S DOXO + CYCLO (complete disappearance of multiple s.c. tumor nodules; 90% + regression of massive intraabdominal tumor on computed tomography scan).</td>
<td></td>
</tr>
<tr>
<td>5 Extrapulmonary small-cell</td>
<td>S DOXO, S L-PAM</td>
<td>S CYCLO + MTX + 5-FU (complete disappearance of malignant pleural effusion).</td>
<td></td>
</tr>
<tr>
<td>6 Breast</td>
<td>S HN2, R 5-FU</td>
<td>S CYCLO + DOXO + VNCR</td>
<td></td>
</tr>
<tr>
<td>7 Acute lymphoblastic leukemia</td>
<td>S DEX</td>
<td>S PRED + VNCR + asparaginase</td>
<td></td>
</tr>
<tr>
<td>8 Acute lymphoblastic leukemia</td>
<td>S DEX</td>
<td>S PRED + VNCR + asparaginase</td>
<td></td>
</tr>
<tr>
<td>9 Breast</td>
<td>R HN2, DOXO, 5-FU; S HN2 + DOXO + 5-FU</td>
<td>S CYCLO + DOXO + 5-FU (dramatically improved liver spleen scan and alkaline phosphatase).</td>
<td></td>
</tr>
<tr>
<td>10 Small-cell lung</td>
<td>S DOXO</td>
<td>R DOXO</td>
<td></td>
</tr>
<tr>
<td>11 Small-cell lung</td>
<td>S HN2</td>
<td>R CYCLO + DOXO + VNCR</td>
<td></td>
</tr>
<tr>
<td>12 Pancreas</td>
<td>R DOXO</td>
<td>R CYCLO + DOXO + VNCR</td>
<td></td>
</tr>
<tr>
<td>13 Small-cell lung</td>
<td>R 5-FU, R DOXO</td>
<td>R 5-FU + DOXO + MIT C</td>
<td></td>
</tr>
<tr>
<td>14 Breast</td>
<td>R HN2, R DOXO</td>
<td>R CYCLO + DOXO + VNCR</td>
<td></td>
</tr>
<tr>
<td>15 Melanoma</td>
<td>R DOXO</td>
<td>R DOXO + VNCR</td>
<td></td>
</tr>
<tr>
<td>16 Diffuse histiocytic lymphoma</td>
<td>R HN2</td>
<td>R CYCLO + VNCR + MTX + ara-C</td>
<td></td>
</tr>
<tr>
<td>17 Esophagus (adenocarcinoma)</td>
<td>R 5-FU, DOXO</td>
<td>R 5-FU + DOXO + MIT C</td>
<td></td>
</tr>
<tr>
<td>18 Lung (adenocarcinoma); pretreatment</td>
<td>R HN2, R DOXO</td>
<td>R CYCLO + DOXO + MTX + PROCARB</td>
<td></td>
</tr>
<tr>
<td>19 Lung (adenocarcinoma; reassay after treatment)</td>
<td>R HN2, R DOXO</td>
<td>R CYCLO + DOXO + MTX + PROCARB</td>
<td></td>
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<tr>
<td>20 Colon</td>
<td>R 5-FU</td>
<td>R CYCLO + MTX + VNCR; R VP-16</td>
<td></td>
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<tr>
<td>21 Ovary</td>
<td>R HN2, R L-PAM, R DOXO</td>
<td>R CYCLO + DOXO</td>
<td></td>
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<tr>
<td>22 Lung (adenocarcinoma)</td>
<td>R DOXO</td>
<td>R DOXO</td>
<td></td>
</tr>
<tr>
<td>23 Multiple myeloma</td>
<td>R L-PAM (not assayed DEX)</td>
<td>R L-PAM + DEX</td>
<td></td>
</tr>
<tr>
<td>24 Pancreas</td>
<td>R DOXO</td>
<td>R 5-FU + DOXO + MIT C</td>
<td></td>
</tr>
<tr>
<td>25 Small-cell lung</td>
<td>R L-PAM, R DOXO</td>
<td>R CYCLO + DOXO + VNCR</td>
<td></td>
</tr>
<tr>
<td>26 Multiple myeloma</td>
<td>R L-PAM, R DEX</td>
<td>R L-PAM + VNCR</td>
<td></td>
</tr>
<tr>
<td>27 Lung (adenocarcinoma)</td>
<td>R DOXO, R L-PAM</td>
<td>R CYCLO + DOXO + MTX + PROCARB</td>
<td></td>
</tr>
<tr>
<td>28 Lung (adenocarcinoma)</td>
<td>R DOXO, R L-PAM</td>
<td>R CYCLO + DOXO + MTX + PROCARB</td>
<td></td>
</tr>
<tr>
<td>29 Diffuse histiocytic lymphoma</td>
<td>R L-PAM, DEX, DOXO</td>
<td>R CYCLO + DOXO + VNCR + PRED</td>
<td></td>
</tr>
<tr>
<td>30 Multiple myeloma</td>
<td>R L-PAM, R DEX</td>
<td>R L-PAM + PRED</td>
<td></td>
</tr>
<tr>
<td>31 CLL</td>
<td>R L-PAM (not assayed DEX)</td>
<td>R L-PAM + DEX</td>
<td></td>
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<tr>
<td>32 Small-cell lung</td>
<td>R L-PAM, R HN2, R DOXO</td>
<td>R CYCLO + MTX + VNCR; R CCNU + DOXO + PROCARB</td>
<td></td>
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<tr>
<td>33 Multiple myeloma</td>
<td>R L-PAM, R DEX</td>
<td>R L-PAM + PRED</td>
<td></td>
</tr>
<tr>
<td>34 Lung (adenocarcinoma)</td>
<td>R HN2, R DOXO</td>
<td>R CYCLO + DOXO + MTX + PROCARB</td>
<td></td>
</tr>
<tr>
<td>35 Lung (adenocarcinoma)</td>
<td>R HN2, R DOXO</td>
<td>R CYCLO + DOXO + MTX + PROCARB</td>
<td></td>
</tr>
<tr>
<td>36 Ovary</td>
<td>R L-PAM, R HN2, R DOXO</td>
<td>R CYCLO + DOXO</td>
<td></td>
</tr>
<tr>
<td>37 Lung (adenocarcinoma)</td>
<td>R DOXO</td>
<td>R DOXO</td>
<td></td>
</tr>
<tr>
<td>38 Multiple myeloma</td>
<td>R L-PAM, R DEX</td>
<td>R L-PAM + PRED</td>
<td></td>
</tr>
<tr>
<td>39 Diffuse histiocytic lymphoma</td>
<td>R DOXO</td>
<td>R CYCLO + DOXO + VNCR + PRED; S DOXO + bleomycin + vinblastine + DTIC (50% decrease in cervical lymphadenopathy for 2 weeks before disease progression).</td>
<td></td>
</tr>
</tbody>
</table>

* S, sensitive to; DOXO, doxorubicin; CYCLO, cyclophosphamide; R, resistant to; L-PAM, L-phenylalanine mustard (melphalan); DEX, dexamethasone; MTX, methotrexate; PRED, prednisone; VNCR, vincristine; MIT C, mitomycin C; PROCARB, procarbazine; CCNU, 1-(2-chloroethyl)-3-cyclohexyl-1-nitrosourea; DTIC, (4-dimethyltriazeno)imidazole-4-carboxamide.
specimen. This unique ability to determine which specific cells are being killed could be of great value in studies on immuno-therapy and immunochemotherapy. Since the assay uses a liquid culture medium, it should be very feasible to design certain types of drug-scheduling experiments, since additional drugs may be added to the culture medium and then washed out after the initial period of exposure to the first drug.

In spite of the above advantages, the assay described here does have some existing and potential limitations. First, the interpretation of the assay depends absolutely upon the observer being able to correctly identify living tumor cells present in the final stained preparation. This is generally easy to do in preparations from solid tumor specimens, where the vast majority of living non-tumor cells are histiocyes and lymphocytes. On the other hand, distinguishing tumor cells from benign mesothelial cells (which are often present in effusions) may be a major problem for an observer without a background in cytotechnology. For this reason, it may be most practical to limit assays on effusions to those samples in which the tumor cells are easily identifiable in the original specimen. Finally, a most important question relating to the presently described assay is the accuracy with which it is capable of predicting the response to cancer chemotherapy in vivo. The assay data revealed a strong correlation between the in vitro chemosensitivity of different types of tumors and the known in vivo response patterns of these tumors. This suggests that the assay could prove useful in new drug screening. Individual clinical correlations, though encouraging, were of a preliminary nature. Further work will be required to determine the accuracy and limitations of the assay.

In the time since we originally submitted this paper, we have modified the assay by basing the results on the ratio of living tumor cells over simultaneously counted duck RBCs. These latter cells are added to the cultures as an internal standard to modify the assay by basing the results on the ratio of living tumor cells. This suggests that the assay is the accuracy with which it is capable of predicting the response of different types of tumors and the known in vivo chemosensitivity. For this reason, it may be most practical to limit assays on effusions to those samples in which the tumor cells are easily identifiable in the original specimen. Finally, the latter cells are added to the cultures as an internal standard to modify the assay by basing the results on the ratio of living tumor cells.

### Table 4

<table>
<thead>
<tr>
<th>Drug</th>
<th>Sensitive</th>
<th>Intermediate</th>
<th>Resistant</th>
</tr>
</thead>
<tbody>
<tr>
<td>Doxorubicin</td>
<td>0.4 µg/ml &lt;30% survival</td>
<td>1.2 µg/ml &lt;30% survival</td>
<td>1.2 µg/ml &gt;30% survival</td>
</tr>
<tr>
<td>Nitrogen mustard</td>
<td>0.7 µg/ml &lt;30% survival</td>
<td>3.5 µg/ml &lt;30% survival</td>
<td>3.5 µg/ml &gt;30% survival</td>
</tr>
<tr>
<td>Melphalan</td>
<td>2.5 µg/ml &lt;30% survival</td>
<td>12.5 µg/ml &lt;30% survival</td>
<td>12.5 µg/ml &gt;30% survival</td>
</tr>
<tr>
<td>Carmustine</td>
<td>11 µg/ml &gt;30% survival</td>
<td>33 µg/ml &gt;30% survival</td>
<td>33 µg/ml &gt;30% survival</td>
</tr>
<tr>
<td>Cis-Platin</td>
<td>1 µg/ml (C) &lt;30% survival</td>
<td>3.3 µg/ml (C) &lt;30% survival</td>
<td>3.3 µg/ml (C) &gt;30% survival</td>
</tr>
<tr>
<td>Mitomycin C</td>
<td>2.5 µg/ml &lt;30% survival</td>
<td>0.5 µg/ml (C) &lt;30% survival</td>
<td>0.5 µg/ml (C) &gt;30% survival</td>
</tr>
<tr>
<td>Vinblastine</td>
<td>0.16 µg/ml (C) &lt;30% survival</td>
<td>0.53 µg/ml (C) &lt;30% survival</td>
<td>0.53 µg/ml (C) &gt;30% survival</td>
</tr>
<tr>
<td>Dexamethasone</td>
<td>0.4 µg/ml (C) &lt;30% survival</td>
<td>1.2 µg/ml (C) &lt;30% survival</td>
<td>1.2 µg/ml (C) &gt;30% survival</td>
</tr>
<tr>
<td>5-FU</td>
<td>10 µg/ml (C) &lt;30% survival</td>
<td>25 µg/ml (C) &lt;30% survival</td>
<td>25 µg/ml (C) &gt;30% survival</td>
</tr>
<tr>
<td>VP-16</td>
<td>125 µg/ml &lt;30% survival</td>
<td>ND</td>
<td>125 µg/ml &gt;30% survival</td>
</tr>
</tbody>
</table>

*Drug exposures are for 1 hr, except for those labeled (C), which are continuous, 4-day exposures. ND, not determined.

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