A Tissue Culture Cytotoxicity Test for Large-Scale Cancer Chemotherapy Screening*

IRVING TOPLIN

(John L. Smith Memorial for Cancer Research, Chas. Pfizer & Co., Inc., Maywood, N.J.)

Recent reviews of the role of tissue culture in cancer chemotherapy screening (5, 6) have emphasized the need for a more extensive trial of cell cultures as a screening tool for the detection of anti-tumor substances.

Eagle and Foley (2-4) have reported the cytotoxic activity against human cell cultures of more than 180 selected compounds. These compounds had previously been tested against at least three experimental animal tumors. It was found that 79 per cent of the substances active against two or more experimental tumors showed cell culture activity at $10^{-4}$ gm/ml or less. This degree of sensitivity was achieved at the cost of 21 per cent of the tumor-negative compounds also exhibiting cell culture activity at the arbitrary cut-off point of $10^{-4}$ gm/ml or less. It was also observed that there was no regular difference in the susceptibility to the various drugs of the cell lines derived from malignant and normal tissue. Eagle and Foley concluded that a large-scale investigation of the usefulness of human cell cultures as a cancer chemotherapy screening procedure was warranted.

Nitta (7-9) has tested 38 antibiotics and synthetics of varying anti-tumor activity against HeLa cell cultures. It was found that, in general, the strong tumor-inhibitory antibiotics were destructive to HeLa cells at the lowest concentrations, while those antibiotics inactive against animal tumors were least injurious to the cell cultures. Nitta concluded that the test method with the use of HeLa cells can be practically applied to the screening or assay of the anti-tumor activity of substances.

The large-scale screening program of the Cancer Chemotherapy National Service Center (CCNSC) with experimental animal tumors affords a unique opportunity to test concomitantly the reliability and sensitivity of a cell culture screen in detecting anti-tumor agents from a large random group of test candidates. This report describes a simple, rapid, and inexpensive tissue culture cytotoxicity test used in this laboratory to supplement the experimental tumors in the screening of fermentation broth filtrates, natural products, and synthetics. Results obtained by this technic for a group of compounds of known tumor activity are also presented. Some preliminary observations are made on the applications and limitations of the test in random screening and in following the fractionation and concentration of anti-tumor agents from crude preparations.

MATERIALS AND METHODS

The tissue culture test used in these studies included three essential steps:

1. Dispensing of graded doses of test solution directly into disposable plastic containers by a microburet technic.

2. Addition of a HeLa or other human cell suspension standardized at 10,000-15,000 cells/ml in growth medium to a total volume of 1 ml/container.

3. Microscopic evaluation of cytotoxicity after 5 days' incubation by a prescribed cytotoxicity rating system.

Details of the procedure were as follows:

Preparation of cell suspension.—Stock cultures of the HeLa and other human cell strains were grown on the glass surface of Blake bottles in Eagle's medium (1) with 10 per cent pooled human serum and 50 units/ml penicillin and 50 $\mu$g/ml dihydrostreptomycin. Stock cultures were refed with fresh medium every 2 days. A suspension of mainly single cells was obtained from a 3-5-day culture by scraping or trypsinizing the cell sheets off the glass and dispersing the cells with repeated pipetting. Cells grown in fluid suspension were also used successfully. In either case, the cell suspension was diluted in fresh medium to a final concentration of 10,000-15,000 cells/ml based on a hemocytometer cell count at about the 100,000 cells/ml level. The cell suspension was placed in a 500-ml. Erlenmeyer flask equipped with a suspended Teflon-coated magnet and with glass and rubber tubing connections leading to a 1-ml. Cornwall syringe (Chart 1). A 3-way adapter placed

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on the syringe allowed recirculation of the medium that remained in the tubing between samples. The suspension was stirred continuously by a low-speed magnetic stirrer.

Preparation and testing of solutions.—Soluble test compounds were dissolved in distilled water or Hanks’ salt solution with, in some cases, addition of small amounts of 0.5 N HCl, 0.5 N NaOH, ethanol, acetone, or methanol to promote solubility. Adjustment to pH 7.2–7.4 was then made when necessary with 0.5 N HCl or 0.5 N NaOH. All solutions and broth filtrates were sterilized by UF-porosity glass filtration. Since only a small quantity of sample was needed for testing, it was convenient to use a 3-ml. Buchner-type UF glass filter flared at the top and seated in a standard 12-ml. centrifuge tube with a rubber O-ring placed under the flare to prevent glass-to-glass contact (Chart 1). Centrifugation for 15 minutes at 2000 r.p.m. was generally sufficient to obtain 1–1.5 ml. of sterile sample.

The culture containers used were the disposable 1.5-ml. vinyl plastic cup trays described by Rightsel and his co-workers (10) growth medium to give the four or more desired test dilutions. HeLa cell suspension at 100,000–150,000 cells/ml in growth medium was added to each dilution at 10 per cent of final volume and thoroughly mixed by repeated pipetting. Two plastic cups were filled by pipette with 1 ml. for each of the test dilutions. As previously, the trays were covered with 2-inch strips of cellophane tape and incubated at 37°C.

Cytotoxicity rating system.—All assays were examined after 5 days’ incubation for evidence of cytotoxicity. Cytotoxicity was judged by direct microscopic examination of the cultures at low-power, 63–100 magnification. The inhibition of cell metabolism as indicated by the phenol red color changes in the growth medium was taken as a secondary confirmation of cytotoxicity.

For microscopic evaluation of cell damage, an arbitrary scale of increasing cytotoxicity based on morphological characteristics was employed.

The main criteria for the rating system were:

<table>
<thead>
<tr>
<th>Grade</th>
<th>Definition</th>
</tr>
</thead>
<tbody>
<tr>
<td>0</td>
<td>Cells firmly adhering to the plastic with clear details, and forming a sheet-like monolayer.</td>
</tr>
<tr>
<td>1</td>
<td>Cell growth inhibited, but majority of cells adhering to the plastic; some granulation and rounding up of cells.</td>
</tr>
<tr>
<td>2</td>
<td>Cells suspended and generally clumped, with pronounced granularity and loss of cell detail; increasing cellular debris.</td>
</tr>
<tr>
<td>3</td>
<td>Mainly single, suspended, shrunken cells with irregular membranes; some disintegrating cell clusters and considerable cellular debris.</td>
</tr>
<tr>
<td>4</td>
<td>Complete cytolysis; all debris.</td>
</tr>
</tbody>
</table>

Two cups were filled per dilution. A distilled-water control and a known-positive control were run with each test series. The plastic trays were covered with 2-inch strips of Scotch cellophane tape with care taken to form an air-tight seal around each cup. Incubation of the cultures was at 37°C.

Testing of insoluble compounds.—Insoluble compounds were suspended in a small amount of ethanol for 1/4 hour prior to the addition of sterile water or Hanks’ salt solution to final volume. The sterile suspension was serially diluted in test tubes in

as being suitable for cell cultures. The trays were prepared as suggested by immersion in 95 per cent denatured alcohol for 1/4 hour and were dried in the bacteriological hood under UV light. The sterile test sample was dispensed directly into the plastic cups in graded volumes with a syringe microburet and syringes calibrated at 0.2 ml/unit of the microburet dial. A 30-gauge blunt-tip needle attached to the syringe allowed accurate placement of the aliquots into the cups (Chart 1). Cell suspension was then added to each cup to a total volume of 1 ml. For routine testing, a half-log dilution schedule was used with dilutions of 1/10, 1/32, 1/100, and 1/320.

Two cups were filled per dilution. A distilled-water control and a known-positive control were run with each test series. The plastic trays were covered with 2-inch strips of Scotch cellophane tape with care taken to form an air-tight seal around each cup. Incubation of the cultures was at 37°C.

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CHART 1.—Schematic diagram of experimental equipment

1 Micro-Metric Instrument Co., Cleveland, Ohio.
significant cellular degeneration; and a lethal end-
point (LE), or concentration causing complete cell
disintegration. In terms of the cytotoxicity scale,
the CE was the lowest concentration of drug that
resulted in a rating greater than one scale unit
over the control culture rating. With the control
cultures of acceptable assays rated at 0 to ½ on the
scale, the CE (1½–2 rating) was generally charac-
terized by rounding and clumping of the cells in the
medium with granulation, loss of cellular detail,
and increased cellular debris. The LE was taken
as the lowest concentration of drug giving a 3½–4
rating.

After 3–5 days’ incubation, the growth medium
of the cell cultures that contained lethal or cyto-
toxic concentrations of test substance usually,
though not always, remained at the original pH of
the medium (pH 7.2–7.4) or drifted to a slightly
more alkaline condition, as shown by the phenol
red indicator. Inactive substances did not prevent
the normal change in medium pH toward the acid
range during the incubation period. The cytotoxic
activity as determined by this pH effect was re-
corded as (+) for the test substance if a definite
inhibition of acid production was observed in any
of the test dilutions; (±) if this effect was slight;
and (−) if the dilution series indicated no inhibi-
tion of acid production as compared with the con-
trol cultures.

In the screening of fermentation broths, it was
observed that some substances manifested cyto-
toxic activity only after 3–4 days’ incubation. Dur-
ing this interval the cultures metabolized
vigorously, and as a result, pH observations were
poor guide to cytotoxic concentrations. There-
fore, microscopic examination of test cultures was,
in all cases, the prime means of judging cyto-
toxicity.

Staining of cultures.—In addition to the direct
rating of cell damage, staining of the test cultures
for detailed study was possible by insertion of
sterile 12-mm, circular glass coverslips into the
plastic cups before an assay series was begun. The
cells rapidly adhered to the glass bottom formed
by the coverslips. The coverslips were easily re-
moved for staining when desired.

RESULTS AND DISCUSSION

A group of compounds of known tumor activity
have been tested by this technic, and the results
are summarized in Table 1. Many of the compo-
ses tested by both methods is not un-
expected. However, since both systems employ the same cell
lines, growth medium, and approximately the
same drug-cell contact times, the good over-all
agreement as to the cytotoxic character of the
compounds tested by both methods is not un-
expected.

The morphological changes in the cell cultures
upon which the cytotoxicity rating scale is based
are the progressive symptoms of cellular degenera-
tion most frequently observed with cytotoxic sub-
stances. Nitta (8), in studies of the action of anti-
tumor antibiotics on HeLa cells, also noted that
the various agents at suitable concentrations pro-
duced common degenerative effects. However, the
cytotoxic action of certain agents tested in our
laboratory could not be categorized by the cyto-
toxicity scale. Certain drugs strongly inhibited cell
growth at concentrations far below those that re-
sulted in the common cytotoxic effects. The cell
cultures appeared “frozen” on the plastic surface,
although cell growth and metabolism, as indicated
### TABLE 1

**CYTOTOXICITY OF VARIOUS KNOWN COMPOUNDS**

| NSC no. | Name | Cytotoxic end-point (μg/ml) | Lethal end-point (μg/ml) | Activity by pH | Eagle & Foley*  
|---------|------|---------------------------|------------------------|--------------|----------------- 
| 4       | 2-Amino-5-nitrothiazole | >60             | >60                  | -        | >100             
| 145     | Phenylphosphonic acid  | >100            | >100                | -        | >100             
| 185     | Actidione              | 0.5–3           | 30–60               | +        | 0.1–1           
| 423     | 2,2-Dichlorophenoxyethanol | 20–100   | 100–200             | ±, ±    | 1–10             
| 426     | 1,2-Diethylene glycol  | >100            | >100                | -        | >100             
| 427     | 2-Mercaptotriophosphonic acid | >100      | >100                | -        | >100             
| 441     | 2-Azido-2-methylpropanol | >100         | >100                | -        | >100             
| 552     | 3-Methoxypropylamine  | >100            | >100                | -        | >100             
| 759     | Aminopterin            | 1–10           | >50                 | +, ±    | 0.01–0.1        
| 742     | Amethopterin           | 1–5            | >100                | +, ±    | 0.01–0.1        
| 743     | Azaserine              | 0.5–2          | 2–10                | +        | 0.1–1           
| 744     | 2,6-Diaminopurine      | 7–20           | 20–60               | +        | 1–10             
| 746     | Urethane               | ≥2000-10,000   | ≥10,000             | -        | >100             
| 747     | N-Methyl acetamide     | >1000          | >1000               | -        | >100             
| 748     | Formamide              | >1000          | >1000               | -        | >100             
| 749     | 8-Azaxazine            | 2–10           | 40–100              | +        | 0.1–1           
| 752     | 6-Thioguanine          | 2–10           | 60–100              | +        | 0.1–1           
| 754     | 2-6-Thiophenalanine    | 3800–1200      | 1800–3000           | +        | >100             
| 755     | 6-Mercaptopurine        | 20–100         | >100                | +        | 0.1–1           
| 756     | 8-Azaanthine           | >60            | >60                 | -        | 10–100          
| 757     | D-Glucoamine hydrochloride | >100    | >100                | -        | >100             
| 759     | Benzimidazole          | >100            | >100                | -        | >100             
| 761     | Methyl 8-pyridyl ketone (3-acetyl pyridine) | >100       | >100                | -        | >100             
| 762     | Nitrogen mustard       | 1–5            | 5–20                | +        | 0.1–1           
| 763     | Dimethyl sulfoxide     | >100            | >100                | -        | >100             
| 805     | Meconic acid (3-hydroxy-4-oxo-4-H-pyran-2,6-dicarboxylic acid) | >100     | >100                | -        | >100             
| 8112    | Cyclopropenecarboxylic acid | >100      | >100                | -        | >100             
| 1487    | N-Butyl thiocyanate    | 20–100         | 40–600              | +        | 10–100          
| 1481    | N-Lauryl thiocyanate   | 6–40           | 40–100              | +        | 0.1–1           
| 1552    | 2,4-Dinitrophenol      | 20–40          | >200                | +, ±    | 10–100          
| 1571    | Resorcinol             | 2000–10000     | 1600–10,000         | +        | >100             
| 1578    | 3-Pyridine sulfonic acid, sodium salt | >100    | >100                | -        | >100             
| 1589    | 5-Pyrimidinecarboxylic acid, 1,2,3,4-tetrahydro-2,4-dioxomonomonohydrate | >60  | >60                 | -        | >100             
| 1697    | p-Acetaminom-anisidine | >100            | >100                | -        | >100             
| 1698    | 2-Hydroxy-4,6-dimethyl pyrimidine hydrochloride | >100 | >100                | -        | >100             
| 2038    | 8-Hydroxyquinoline     | 0.2–1          | 0.2–7               | +        | 1–10             
| 2059    | 6-Diethylaminomethyl chloride hydrochloride | 10–60 | 60–200             | +        | 1–10             
| 2193    | 6-Benzoylpurineic acid  | >100            | >100                | -        | >100             
| 2196    | Nitrofurazone          | 7–60           | ≥20–60              | +        | 1–10             
| 2107    | Furadantin             | 10–60          | 100–200             | +        | >100             
| 2559    | N-Phenylsuccinimide    | >100            | >100                | -        | >100             
| 2587    | 2,4-Sulfonyldiphenol  | 20–60          | >60                 | ±        | 10–100          
| 2626    | Methyl chloroacetate   | 10–30          | 30–200              | +        | 10–100          
| 2652    | 2-Dimethylaminomethanol | >100       | >100                | -        | >100             
| 2675    | Tri-n-butyl phosphate  | >100            | >100                | -        | >100             
| 2773    | N-2-Hydroxyethylphthalimid | >100  | >100                | -        | >100             
| 2852    | 2-Naphthalonic acid, 3-hydroxy-7-sulfo- | >100 | >100                | -        | >100             
| 2853    | Benzoic acid, m-sulfo- | >100            | >100                | -        | >100             
| 3051    | N-Methyl formamide     | >100            | >100                | -        | >100             
| 3053    | Actinomycin D          | 0.01–0.04      | 0.1–0.5             | +        | 0.01–0.1        
| 3054    | Methyldimethane        | >100            | >100                | -        | >100             
| 3055    | Pyromycin dihydrochloride | 0.1–0.5      | 0.5–3               | +        | 0.1–1           
| 3057    | 8-Aza-2,6-diaminopurine sulfamate | >60    | >60                 | -        | >100             
| 3060    | Potassium arsenate     | 3–10           | 10–20               | +        | 0.1–1           
| 3061    | Daraprim, pyrimethamine | 90–60     | >60                 | +        | 0.1–1           
| 3067    | Netropsin hydrochloride | 30–100     | 100–300             | +        | 10–100          
| 3075    | 4,6-Diamino-1-(p-carboxyphenyl)-1,2-dihydro-2,2-dimethyls-triazine, hydrochloride | >60  | >60                 | -        | >100             
| 3076    | 4-Amino-6-aminon-1,2-dihydro-2-phenyl-s-triazine | 6–20   | ≥200               | +        | 1–10             
| 3081    | 4,6-Diamino-1-(4'-chlorophenyl)-2-n-hexyl-1,2-dihydro-2-triazine hydrochloride | 10–60 | 60–200           | +        | 1–10             
| 3083    | 4,6-Diamino-1-(3'-chlorophenyl)-1,2-dihydro-2-n-hexyl-1,2-dihydro-2-triazine hydrochloride | 15–40 | 60–200          | +        | 1–10             
| 3087    | Oxophenarsine hydrochloride | 6–20   | 30–100            | +        | 1–10             
| 3089    | Methylened blue chloride | 1–3       | 10–30              | +        | 0.1–1           

* References 2, 5, 4.

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### TABLE 1: ADDENDUM

**CYTOTOXICITY OF VARIOUS KNOWN COMPOUNDS**

(Additions and revisions as of September 1, 1959)

<table>
<thead>
<tr>
<th>NSC no.</th>
<th>Name</th>
<th>Cytotoxic end-point (μg/ml)</th>
<th>Lethal end-point (μg/ml)</th>
<th>Activity by pH</th>
<th>Eagle &amp; Foley* ID₅₀ (μg/ml)</th>
</tr>
</thead>
<tbody>
<tr>
<td>643</td>
<td>Benzene sulfonhydrazide</td>
<td>&gt;100</td>
<td>≥100</td>
<td>-</td>
<td>&gt;100</td>
</tr>
<tr>
<td>741</td>
<td>Hydrocortisone</td>
<td>40-120</td>
<td>≥400</td>
<td>+, ±</td>
<td>&gt;100</td>
</tr>
<tr>
<td>750</td>
<td>Myleran</td>
<td>160-600</td>
<td>≥600</td>
<td>+</td>
<td>&gt;100</td>
</tr>
<tr>
<td>2105</td>
<td>Isopropyl N-phenyl carbamate</td>
<td>500-1600</td>
<td>1600-5000</td>
<td>+, ±</td>
<td>&gt;100</td>
</tr>
<tr>
<td>3056</td>
<td>Puromycin aminonucleoside</td>
<td>100-500</td>
<td>≥500</td>
<td>+</td>
<td>&gt;100</td>
</tr>
<tr>
<td>3058</td>
<td>Benzotriazole</td>
<td>160-400</td>
<td>1200-5000</td>
<td>+</td>
<td>&gt;100</td>
</tr>
<tr>
<td>3098</td>
<td>Chloramphenicol</td>
<td>&gt;100</td>
<td>&gt;100</td>
<td>-</td>
<td>10-100</td>
</tr>
<tr>
<td>3099</td>
<td>Polyc acid</td>
<td>&gt;1000</td>
<td>&gt;1000</td>
<td>-</td>
<td>&gt;100</td>
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<tr>
<td>3085</td>
<td>DL-Desthiobiotin</td>
<td>&gt;1000</td>
<td>&gt;1000</td>
<td>-</td>
<td>&gt;100</td>
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<tr>
<td>3097</td>
<td>1,5-Diaminobiuret</td>
<td>80-250</td>
<td>≥500</td>
<td>-</td>
<td>10-100</td>
</tr>
<tr>
<td>3089</td>
<td>Galactosflavin</td>
<td>250-1600</td>
<td>1600-5000</td>
<td>+</td>
<td>&gt;100</td>
</tr>
<tr>
<td>4339</td>
<td>4-(p-Dimethylaminostyryl)quinoline</td>
<td>6-40</td>
<td>40-120</td>
<td>+</td>
<td>0.1-1</td>
</tr>
<tr>
<td>4731</td>
<td>4-Ethylamino-1,3,4-thiadiazole</td>
<td>300-940</td>
<td>1500-3000</td>
<td>+</td>
<td>&gt;100</td>
</tr>
<tr>
<td>5904</td>
<td>4-Amino-6-anilino-1,2-dihydro-2,2-dimethyl-</td>
<td>800-780</td>
<td>1000-2500</td>
<td>+, ±</td>
<td>&gt;100</td>
</tr>
<tr>
<td>5956</td>
<td>Narcotine</td>
<td>8-50</td>
<td>80-250</td>
<td>+</td>
<td>1-10</td>
</tr>
<tr>
<td>20971</td>
<td>Cytoxan</td>
<td>&gt;200</td>
<td>&gt;200</td>
<td>-</td>
<td></td>
</tr>
</tbody>
</table>
by acid production, had ceased. For these and any other cultures over which there was any doubt concerning viability, a recovery step was added to the test procedure. After 5 days' incubation, the drug solution was removed, and fresh growth medium was added to the cell cultures. The cups were resealed and incubated for an additional 3–5 days. Microscopic examination of the cultures for the presence or absence of actively multiplying cells allowed accurate pinpointing of lethal drug concentrations.

The tissue culture test described in this report is currently used in our laboratory as an auxiliary screen to the experimental animal tumors for the detection of potential anti-tumor substances. It has proved to be a rapid, inexpensive, and reproducible method for determining the cytotoxicity of large numbers of samples. A well trained technician can easily perform and evaluate 30 assays daily; that is, 30 samples at four dilutions in duplicate. This includes the routine maintenance of stock cultures and other associated duties. In addition, the test serves as an assay method for anti-tumor activity that is a valuable aid in the fractionation of crude preparations that show experimental tumor and cell culture activities. Tis-

![Chart 2](chart2.png)

**Chart 2.**—Curves showing cytotoxicity scale rating as a function of drug concentration for four selected compounds. The readings were made on HeLa cell cultures after 5 days incubation at 37°C.

![Figs. 1-5](figs1-5.png)

**Figs. 1-5.**—Photomicrographs of HeLa cells showing typical appearance of cultures for each grade of the cytotoxicity rating scale. X300. The grades for the figures are 0, 1, 2, 3, and 4, respectively.
sue culture results are constantly monitored by the animal tumor tests. It has been our experience that cell culture activity is a reliable measure of the degree of concentration achieved in a chemical operation on a cytotoxic anti-tumor broth filtrate. In many cases, a preliminary evaluation of cytotoxic activity after only 3 days' incubation is sufficient for determining the effectiveness of a particular treatment.

SUMMARY

A simple, rapid, and relatively inexpensive tissue culture cytotoxicity test suitable for large-scale cancer chemotherapy screening has been described. The method involves the microscopic evaluation of cytotoxicity against human cell cultures for varying dilutions of test substance after 5 days' incubation. The results obtained by this technic with a group of known compounds have been given. Some preliminary observations have been made on the applications and limitations of the method in random screening and in following the concentration of anti-tumor agents from crude preparations.

ACKNOWLEDGMENTS

I would like to express my thanks to Dr. Frank M. Schabel, Jr., of the Southern Research Institute for his original suggestions on the practicality of a cytotoxicity test of this type in cancer chemotherapy screening, and to Mr. René Schmitter for technical assistance.

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A Tissue Culture Cytotoxicity Test for Large-Scale Cancer Chemotherapy Screening

Irving Toplin

*Cancer Res* 1959;19:959.

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