Clinical Application of the Thymidine Incorporation Assay*

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ABSTRACT

An improved method for testing human tumors against chemotherapeutic agents was developed. Drug effects were quantitated in the thymidine incorporation assay (TIA) by measuring inhibition of deoxyribonucleic acid (DNA) synthesis by the proliferating cell population following exposure to anticancer drugs. Results were obtained within five days. A total of 480 out of 568 tumors received were evaluable for an overall growth rate of 85 percent. Even small biopsy specimens could be successfully grown. In 126 correlations with clinical response, the TIA had a prediction accuracy for sensitivity of 51 percent and a prediction accuracy for resistance of 97 percent. Because it is rapid, has a high growth rate, and is not subject to clumping and other cellular artifacts, the TIA represents a significant improvement over colony counting assays for predicting drug effects in human tumors.

Introduction

Studies from a number of laboratories have shown that predictive chemosensitivity tests are highly correlated with clinical course in patients with cancer.3,8,11,12 Response rates are higher when patients are treated on the basis of assay results compared to "physician's choice" and survival may be increased even in patients with refractory disease.1,13 The most often used chemosensitivity assay is the clonogenic assay, often referred to as the Human Tumor Colony-forming Assay (HTCFA) or the Human Tumor Stem Cell Assay.9,10 However, the usefulness of the HTCFA for determining activity of anti-cancer agents against human tumors is limited by several technical problems. It is diffi-
cult to obtain perfect single cell suspensions from solid tumors. Consequently, cell clumps present at the outset of most experiments lead to inaccurate assessment of tumor cell growth and drug sensitivity. The HTCFA requires 14 to 21 days for completion, and therapy is often delayed while waiting for assay results. Finally, because of low cloning efficiencies only 25 to 60 percent of most tumors can be successfully grown in the HTCFA. To overcome some of the technical difficulties of the HTCFA and to make chemosensitivity testing more readily available, the rapid thymidine incorporation assay reported here was developed by the present authors.

**Materials and Methods**

**Tumor Types**

A total of 568 tumors was received consisting of the following histologically confirmed types (number received): breast carcinoma (42), colon carcinoma (51), lung carcinoma (34), melanoma (82), multiple myeloma (36), ovarian carcinoma (58), sarcoma (138), and miscellaneous (127).

**Preparation of Tumors**

The preparation and enzymatic digestion of tumors used in this study were identical to that reported for the HTCFA. Briefly, connective and adipose tissues and areas of necrotic tumor were removed from surgical specimens. Tumors were weighed and minced into pieces less than two mm in diameter in the presence of Roswell Park Memorial Institute (RPMI) 1640 medium containing 15 percent heat-activated fetal calf serum (FCS). Tumors were washed twice with this solution.

**Enzymatic Digestion**

Tumor fragments were placed in 125 ml trypsinizing flasks. Prewarmed RPMI 1640 containing 0.01 percent DNase I (2650 Kunitz units per mg) and 0.14% collagenase Type I were added at a volume of 30 ml for each g of tissue. After digestion at 37°C for 90 min, the free cells were passed through a 100 μ nylon mesh and centrifuged at 200 × g for 10 min. The supernatant was removed and the cells were resuspended in RPMI 1640 supplemented with 15 percent FCS, 0.3 μg per ml L-glutamine, 100 U per ml penicillin, 100 μg per ml streptomycin, and 1.25 μg per ml Fungizone. The cells were centrifuged again and the pellet was resuspended in supplemented RPMI 1640. Cell yield and viability were determined by trypan blue exclusion. A 10 μl sample of the pellet was transferred to a clean glass slide for preparation of a smear. The slide was allowed to air-dry and then was stained with Wright solution followed by Giemsa counter stain.

**Assay System**

An underlayer of 0.5 percent agarose was prepared by mixing 3.5 ml of molten three percent agarose with 16.5 ml of RPMI 1640 and adding 0.5 ml of this mixture to each 16 × 18 mm well of a 24-well plate. The agarose was allowed to set for 10 min at 4°C. Cells were suspended in 0.4 percent agarose in supplemented RPMI 1640, and 0.5 ml of this mixture was added to each well for a final viable cell concentration of 6 × 10⁴ cells per well. Plates were refrigerated for 10 min at 4°C. Drugs were added as a liquid

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* Flow Laboratories, McLean, VA.
overlay at a volume of 50 μl. Mercuric chloride added at a final concentration of 100 μg per ml of three wells served as the positive control. Plates were incubated at 37°C in a six percent CO₂ atmosphere. After 72 hours, 5 μCi of tritiated thymidine (specific activity 2.0 Ci per mM)1 were layered over each well and the plates returned to the incubator for an additional 36 to 48 hours.

Incorporation of thymidine was terminated by transferring the agarose layers from each well to 15 ml centrifuge tubes,** bringing the volume up to 11 ml with phosphate buffered saline (PBS) and boiling the tubes for 30 min in a waterbath. The tubes were centrifuged, the pellets washed twice with three ml PBS, dissolved in 0.3 ml of 0.075 N KOH, and transferred to scintillation vials containing five ml Optifluor.†† Radioactivity in each vial was measured in a Beckman liquid scintillation counter.‡‡ An assay was considered valid if the average count of the untreated control was greater than 300 cpm, the CV was less than 50 percent, and the positive control showed at least 80 percent inhibition of thymidine uptake when compared to the untreated controls.

** New England Nuclear, Boston, MA.
** No. C3051-870, Beral Scientific, Arleta, CA.
†† Packard Instrument Company, Inc., Downers Grove, IL.
‡‡ LS3800, Beckman Instruments, Irvine, CA.

DESIGN OF CLINICAL CORRELATION STUDIES

In vitro sensitivity to an anticancer drug was defined as ≥80 percent inhibition of thymidine incorporation. Clinical responses to chemotherapy were evaluated by conventional criteria. In vivo sensitivity was defined as either a complete response or a partial response (50 percent or greater decrease in the size of measurable lesions) to therapy for at least one month. Minor responses or stabilization of disease were considered no response. At least a 75 percent decrease in the serum myeloma protein was defined as a response for patients with multiple myeloma.

RESULTS

EVALUABILITY RATE

A total of 568 fresh human tumor biopsies were evaluated for these studies, and 480 (85 percent) were successfully grown in the TIA (table I). Malignant melanomas and carcinomas of the lung and ovary had evaluability rates in excess of 90 percent.

UPTAKE OF LABEL

 Autoradiographs performed on fresh tumor cells exposed to tritiated thymidine showed uptake of the radiolabel by malignant cells, with insignificant uptake by normal cells. Twelve specimens plated in the assay were classified as non-malignant on the pathology report. Two (17 percent) incorporated sufficient label to fulfill the criteria for growth. Both specimens were "uninvolved" lymph nodes from patients with known lymph node involvement with melanoma elsewhere. Upon cytologic review of the smears prepared from these two specimens, the presence of malignant melanoma cells was confirmed.
TABLE I
Evaluability of Fresh Human Tumors with the Thymidine Incorporation Assay

<table>
<thead>
<tr>
<th>Tumor Type</th>
<th>Number Received</th>
<th>Number Evaluable (percent)</th>
<th>Counts Per Minute Median</th>
<th>Counts Per Minute Range</th>
</tr>
</thead>
<tbody>
<tr>
<td>Breast</td>
<td>42</td>
<td>31 (74%)</td>
<td>2,500</td>
<td>360 - 39,000</td>
</tr>
<tr>
<td>Colon</td>
<td>51</td>
<td>39 (76%)</td>
<td>4,150</td>
<td>500 - 180,000</td>
</tr>
<tr>
<td>Lung</td>
<td>34</td>
<td>32 (94%)</td>
<td>3,900</td>
<td>360 - 54,000</td>
</tr>
<tr>
<td>Melanoma</td>
<td>82</td>
<td>76 (93%)</td>
<td>15,600</td>
<td>350 - 200,000</td>
</tr>
<tr>
<td>Myeloma</td>
<td>36</td>
<td>30 (83%)</td>
<td>24,300</td>
<td>678 - 276,000</td>
</tr>
<tr>
<td>Ovarian</td>
<td>58</td>
<td>54 (93%)</td>
<td>4,000</td>
<td>374 - 130,000</td>
</tr>
<tr>
<td>Sarcoma</td>
<td>138</td>
<td>112 (81%)</td>
<td>1,600</td>
<td>330 - 335,000</td>
</tr>
<tr>
<td>Other</td>
<td>127</td>
<td>106 (83%)</td>
<td>2,000</td>
<td>320 - 297,000</td>
</tr>
<tr>
<td>Totals</td>
<td>568</td>
<td>480 (85%)</td>
<td>3,900</td>
<td>320 - 335,000</td>
</tr>
</tbody>
</table>

Plating Density, Labeling Efficiency, and Time Curves

Tritiated thymidine incorporation was looked at as a function of cell density to determine if there was an optimal number of cells to be plated per well. A total of 11 tumors (three melanomas, two lung cancers, two sarcomas and one each of multiple myeloma, breast cancer, colon cancer, and ovarian cancer) were plated at various cell densities and thymidine incorporation was measured. Representative results are shown in table II. Maximum thymidine incorporation was observed when 3 to $6 \times 10^4$ cells per well were plated. Counts per minute were slightly less at $3 \times 10^4$ and $1.5 \times 10^5$ cells per well. Peak labeling efficiencies occurred at 1.5 to $3 \times 10^4$ cells per well. No difference in labeling efficiency versus cell density was noted for the various tumor types. When more than $1.5 \times 10^5$ cells per well were plated, labeling efficiency decreased dramatically. Time course studies of tritiated thymidine uptake showed that incorporation peaked after three to four days and then decreased.

Small Biopsy Specimens

Those solid tumors submitted for chemosensitivity testing weighing \( \leq \) one g were considered in this category. Evaluability rate was directly related to tumor size. While 376/432 (87 percent) of biopsies larger than one g yielded successful assays, 74/100 (74 percent) of specimens one g or less were evaluable. Tumors weighing 0.7 to 1.0 g had an overall growth rate of 85 percent. This percentage dropped to 64 percent for tumors weighing 0.6 g or less. A mini-

TABLE II
Thymidine Incorporation and Labeling Efficiency

<table>
<thead>
<tr>
<th>Cells (10^3) Plated/Well</th>
<th>Thymidine Incorporation (cpm $\pm$ SD)</th>
<th>Labeling Efficiency (cpm/1000 cells)</th>
</tr>
</thead>
<tbody>
<tr>
<td>1.5*</td>
<td>10 $\pm$ 4</td>
<td>6</td>
</tr>
<tr>
<td>3</td>
<td>20 $\pm$ 2</td>
<td>7</td>
</tr>
<tr>
<td>6</td>
<td>135 $\pm$ 14</td>
<td>22</td>
</tr>
<tr>
<td>15</td>
<td>510 $\pm$ 42</td>
<td>34</td>
</tr>
<tr>
<td>30</td>
<td>1150 $\pm$ 56</td>
<td>38</td>
</tr>
<tr>
<td>60</td>
<td>1910 $\pm$ 201</td>
<td>31</td>
</tr>
<tr>
<td>150</td>
<td>1305 $\pm$ 96</td>
<td>8</td>
</tr>
<tr>
<td>300</td>
<td>656 $\pm$ 41</td>
<td>2</td>
</tr>
<tr>
<td>1.5†</td>
<td>12 $\pm$ 6</td>
<td>8</td>
</tr>
<tr>
<td>3</td>
<td>39 $\pm$ 5</td>
<td>12</td>
</tr>
<tr>
<td>6</td>
<td>180 $\pm$ 25</td>
<td>30</td>
</tr>
<tr>
<td>15</td>
<td>645 $\pm$ 38</td>
<td>43</td>
</tr>
<tr>
<td>30</td>
<td>1440 $\pm$ 156</td>
<td>48</td>
</tr>
<tr>
<td>60</td>
<td>1560 $\pm$ 95</td>
<td>26</td>
</tr>
<tr>
<td>150</td>
<td>1508 $\pm$ 102</td>
<td>10</td>
</tr>
<tr>
<td>300</td>
<td>128 $\pm$ 40</td>
<td>0.4</td>
</tr>
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*Ovarian carcinoma
†Lung carcinoma

cpm = counts per minute
SD = standard deviation
mum of $10^5$ viable cells per assay were required for tumor growth. Four or more anticancer agents were tested in 95 percent of all evaluable assays.

**In Vitro/In Vivo Correlations with the TIA**

A total of 126 individual correlations were possible. In 32/63 tests sensitivity *in vitro* corresponded to sensitivity *in vivo*, giving an accuracy for predicting sensitivity of 51 percent. In 61/63 tests resistance *in vitro* corresponded to resistance *in vivo* for a 97 percent accuracy for predicting resistance (table III).

**Discussion**

The clinical usefulness of the Human Tumor Colony-forming Assay is limited by long turnaround time (two to three weeks), low growth rates (25 to 60 percent) for most tumors, and clumping artifacts that can lead to erroneous predictions of chemoresistance. An improved technique was developed by us in which *in vitro* growth is measured by incorporation of tritiated thymidine. Assay results were available within five days with an overall assay success rate of 85 percent. Only proliferating tumor cells, those cells which comprise the growth fraction of the tumor, incorporated radiolabeled thymidine. Thus, the TIA was not subject to the clumping artifacts of the HTCFA. The number of cells per well plated in the TIA was $6 \times 10^4$. This contrasts with the HTCFA in which up to $5 \times 10^5$ cells per well are usually used. Thus, more drugs can be tested per tumor in the TIA than in the HTCFA.

These technological advances make the TIA more practical for routine laboratory use than the HTCFA. However, *in vitro-in vivo* correlations remain the *sine qua non* by which predictive assays are measured. For the TIA, 126 correlations with clinical response were available. Patients whose tumors were predicted by the TIA to be sensitive had a 51 percent response rate. The accuracy of the TIA to predict resistance was 97 percent. The clinical correlations reported here compare favorably with those of the more widely used HTCFA for which the accuracy of predictions of sensitivity have been reported to be 57 to 69 percent,3,8 and predictions of resistance were 80 to 97 percent.8,12 All chemosensitivity assays appear to over-predict sensitivity. This may be due to inappropriate selection of drug exposure times and concentrations *in vitro* as well as to poor drug delivery clinically. In these experiments, peak plasma concentrations of drugs in continuous exposure were used to minimize the possibility that a clinically active drug might be overlooked. The number of false positive assay results (assay predicts sensitivity but the patient fails to respond) may be reduced with refinements of the *in vitro* drug testing conditions while improvements in drug delivery methods may allow anticancer drugs to be more accurately and safely administered in the clinic. For example, highly accurate correlations between *in vitro* sensitivity and clinical response of patients treated by high-dose, intraarterial chemotherapy have recently been reported.5 In the

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</thead>
<tbody>
<tr>
<td>32</td>
<td>31</td>
<td>2</td>
<td>61</td>
</tr>
</tbody>
</table>

*In vitro sensitivity was defined as $> 80$ percent inhibition of thymidine incorporation in drug-treated wells compared to untreated controls. Prediction accuracy for sensitivity: $51% = \text{Sens/Sens}$

Prediction accuracy for resistance: $97% = \text{Res/Res}$

Prediction accuracy for sensitivity: $\text{Sens/Res + Sens/Res}$

Prediction accuracy for resistance: $\text{Res/Res + Res/Res}$
study, all 21 patients with liver metastases whose tumors were sensitive \textit{in vitro} had objective clinical responses.

In evaluating the clinical utility of a predictive assay good clinical correlations are essential but other factors must be taken into consideration.\textsuperscript{4} Results must be available quickly to be useful for clinical decision making. Growth rates must be high enough to assure a reasonable likelihood that the patient subjected to biopsy will benefit from the \textit{in vitro} sensitivity data. Furthermore, an assay system should be applicable to small biopsy specimens from which few tumor cells are obtained. In all these areas, the TIA has been shown to have substantial advantages over the HTCFA. The current status of \textit{in vitro} chemosensitivity testing is not advanced enough to advocate its routine use as a predictive assay for all patients with malignant disease. However, in patients with refractory disease or with metastatic disease from unknown primaries, or in those patients for which there is no standard treatment, chemosensitivity results should be a useful aid to the oncologist in selecting appropriate therapy.

References


