Assessment of *in Vitro* Drug Sensitivity of Human Tumor Cells Using 
$[^3]H$\(^t\)Thymidine Incorporation in a Modified Human Tumor Stem Cell Assay

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**ABSTRACT**

We have developed a method for performing *in vitro* drug testing on primary human tumor explants which is a variant of the human tumor stem cell assay (HTSCA) described previously by Salmon et al. (N. Engl. J. Med., 298: 1321, 1978). The method utilizes a cell-containing liquid top layer and a soft-agar bottom layer. Tumor growth is measured by $[^3]H$\(^t\)thymidine incorporation into material precipitable by 5% trichloroacetic acid. Results show linear correlations with number of cells plated and with number of colonies per plate measured using the HTSCA, when cell aliquots from one sample are used. *In vitro* drug sensitivity, as determined by inhibition of $[^3]H$\(^t\)thymidine incorporation, correlates with HTSCA results in 54 of 61 determinations (89%). Of 22 experiments in which drug sensitivity curves were compared, 21 (95%) were similar in both systems. The $[^3]H$\(^t\)thymidine method yields results more quickly (5 days after samples are plated) and with smaller variances than those from the HTSCA, when cell aliquots from one sample are used. *In vitro* drug sensitivity, as determined by inhibition of $[^3]H$\(^t\)thymidine incorporation, correlates with HTSCA results in 54 of 61 determinations (89%). Of 22 experiments in which drug sensitivity curves were compared, 21 (95%) were similar in both systems. The $[^3]H$\(^t\)thymidine method yields results more quickly (5 days after samples are plated) and with smaller variances than those from the HTSCA, when cell aliquots from one sample are used. *In vitro* drug sensitivity, as determined by inhibition of $[^3]H$\(^t\)thymidine incorporation, correlates with HTSCA results in 54 of 61 determinations (89%). Of 22 experiments in which drug sensitivity curves were compared, 21 (95%) were similar in both systems. 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*methyl-[^H]H*thymidine (6.7 Ci/mmol) was from New England Nuclear, Boston, Mass., diluted with PBS to 75 μCi/ml, and stored at 4°C as a stock solution; hydroxide of Hyamine 10-X (n solution in methanol) was from Packard Instrument Co., Downers Grove, Ill.; and Hydrofluor scintillation fluid was from National Diagnostics, Somerville, N. J.

**Preparation of Solid Tumor Specimens.** Solid tumor specimens all weighed less than 2 g. Within 3 hr of surgical removal, each sample was placed in Mc5G for 0.5 hr to prevent bacterial contamination during sample collection. The specimen was then rinsed in Hanks’ balanced salt solution and placed in a 100-mm Petri dish with 1 ml of Mc5G and mechanically minced for 10 to 20 min with sterile scalpels. The specimen was then passed through a 100-μm mesh stainless steel screen. The residual material on the stainless steel screen was removed steriley, and the screen was washed with Mc5P/S. This mixture was then spun at 150 × g for 10 min and resuspended in the same medium, and cells were counted using a hemocytometer (Neubauer Brite line). This single-cell suspension was refrigerated and later combined with the suspension obtained via enzymatic methods next described.

The residual material on the stainless steel screen was removed steriley, and the screen was washed with Mc5P/S. This mixture was then spun at 150 × g for 10 min and resuspended in 10 ml trypsin stock solution. After a 10-min incubation at 37° in a water bath, the sample was again passed through a 100-μm screen, and the effluent was spun. The supernatant was again removed, and the pellet was resuspended in Mc5P/S and counted. These cells were recombined with the mechanically dissociated cells.

**Preparation of Tumor Effusions and Cell Lines.** Heparinized effusions were passed through 100-μm screens and spun at 150 × g for 10 min. The sediment was then resuspended in Mc5P/S and counted visually. Human cell lines grown to confluence in monolayer culture with Roswell Park Memorial Institute Tissue Culture Medium 1640 and 10% by volume fetal bovine serum were bathed in trypsin stock solution for 5 min. The flasks were washed with Hanks’ solution and the detached cells were spun at 150 × g for 10 min, resuspended in Mc5P/S, and counted. These cells were recombined with the mechanically dissociated cells.

**Preparation of Underlayers.** Enriched McCoys’ Medium 5A, as described by Pike and Robinson (6), was mixed with 10 ml of 3% Trypsinase soy broth, 0.6 ml L-asparagine solution (0.6 mg/ml) in PBS and 0.3 ml DEAE-dextran as described by Salmon et al. (7). This medium was mixed with 3% agar to a final agar dilution of 0.5%, and the mixture was mixed immediately prior to use with DNase I (0.2 mg/ml) in a stock solution. After a 10-min incubation at 37°, the sample was diluted 1 ml was placed in a 35-mm Petri dish, or alternatively, 3 ml were plated in a 60-mm Petri dish. Petri dishes of one diameter were used for each measurement.

**Preparation of Top Layer.** Viable tumor cells, as determined by trypan blue exclusion, were diluted to a concentration of 2.5 × 10⁶ cells per ml. Two-tenths ml cell suspension and 0.8 ml enriched CMRL Medium 1066, as described by Salmon et al. (7) (but without 2-mercaptoethanol), were added to each 35-mm Petri dish on top of the 0.5% agar layer, or alternatively, 0.5 ml cell suspension and 2.5 ml enriched CMRL Medium 1066 were added to the 60-mm dishes. In addition, 0.3% agar upper layers containing tumor cells and enriched CMRL Medium 1066 were added to perform the normal HTSCA.

In the chemosensitivity assays, drugs to be tested were mixed in the top layer at peak plasma concentration, 0.1 and 0.01 peak plasma concentrations (1).

**[^H]H*thymidine Incorporation Assay.** For this assay, plates were incubated for 4 days at 37° in a humidified atmosphere with 7% CO₂. On Day 4, 25 μl of[^H]H*thymidine stock solution were pipetted into the 35-mm liquid top layer dishes, or 75 μl of stock solution were pipetted into the 60-mm dishes. Samples were then incubated an additional 18 to 24 hr.

On Day 5, the liquid top layer dishes were washed with PBS. The washes were spun at 500 × g for 10 min. All subsequent centrifugations in this assay were also spun at 500 × g for 10 min. The supernatant was poured off, and the pellet was resuspended in 5% TCA at 4° for 30 min. The sample was then recenterrifuged, the supernatant was poured off, and the pellet was resuspended in 5% TCA at 4° for another 10 min. This was then spun again, and the pellet was resuspended in methanol and sedimented. The supernatant was poured off, and the pellet was resuspended in 0.5 ml Hyamine hydroxide 10-X. The sample was then heated to 60° in a water bath for 1 hr. The specimen was then placed in 12 ml of Hydrofluor scintillation fluid, and radioactivity incorporated (cpm) was determined using a Packard Tri-Carb liquid scintillation spectrometer or a Beckman Model LS8100 spectrometer.

**HTSCA.** Double agar assay plates were incubated 2 to 4 weeks using the same conditions as the assay described above. The plates were counted either visually or using the Bausch and Lomb Omnicon FAS II optical counter with a colony diameter defined as 80 μm or greater.

Both liquid top layer assay and HTSCA were performed in triplicate for each measurement.

**RESULTS**

**Visual Counting HTSCA versus Liquid Top Layer Assay.** In 6 primary tumors (Table 1), colony growth in the liquid top layer system was compared to that observed on the same day in the HTSCA. Visual counting showed an increase in colonies in the liquid top layer system ranging from 173 to 670%. Samples counted by Bausch and Lomb Omnicon FAS II optical counter supported the increased ratio of liquid to double agar found by visual counting. Discrepancies noted between mean number of colonies per plate determined visually and those determined by the FAS II may relate to the slightly smaller

Table 1

<table>
<thead>
<tr>
<th>Tumor</th>
<th>Double agar</th>
<th>Liquid top</th>
</tr>
</thead>
<tbody>
<tr>
<td></td>
<td>Colony count</td>
<td>Colony count</td>
</tr>
<tr>
<td>Melanoma</td>
<td>20</td>
<td>65</td>
</tr>
<tr>
<td>Soft-part sarcoma</td>
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<td>75</td>
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<td>26</td>
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<tr>
<td>Unclassified sarcoma</td>
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<td>34</td>
</tr>
<tr>
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<td>34</td>
<td>169</td>
</tr>
<tr>
<td>Melanoma</td>
<td>49</td>
<td>151</td>
</tr>
</tbody>
</table>

* Number of plates averaged to give mean value per plate listed under each assay.

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Characteristics of $[3\text{H}]$Thymidine Incorporation into Tumor Cells. Chart 1 demonstrates that the incorporation of $[3\text{H}]$thymidine is directly proportional to the number of viable tumor cells plated over a 3-log range ($10^3$ to $10^6$ cells/plate). In comparing cytology of Day 1 versus Day 4 cytospin preparations grown in the liquid top system, it was noted by a clinical pathologist that tumor cells were present and that the percentage of malignant cells did not decrease over the 4-day interval.

Incorporation to Number of Viable Tumor Cells Plated. Chart 1 shows an example of a primary human tumor expiant plated in the assay incorporated less than 140 cpm/plate. In addition, microscopic examination of the plates failed to reveal evidence of growth.

Comparison of HTSCA Colony Count and $[3\text{H}]$Thymidine Incorporation to Number of Viable Tumor Cells Plated. Chart 2 shows an example of a primary human tumor expiant plated at various cell dilutions in both the HTSCA and the $[3\text{H}]$thymidine incorporation assay. Both the number of colonies per plate and amount of $[3\text{H}]$thymidine incorporation are directly proportional to the number of cells plated over a 2-log range. In the range of $5 \times 10^4$ to $5 \times 10^5$, which was used for drug testing, direct proportionality was demonstrated for both assays in all cases tested. Thus, in this range, the amount of $[3\text{H}]$thymidine incorporated is directly proportional to the number of colonies per plate.

Drug Studies Using HTSCA and $[3\text{H}]$Thymidine Incorporation. In primary human tumor explants and human cell lines where the mean value of control plates in the HTSCA was greater than 50 colonies/plate and where the liquid top layer variant showed $[3\text{H}]$thymidine incorporation of greater than 500 cpm, in vitro drug sensitivity spectra were evaluated. The ratio of radiolabel incorporated into test samples to label incorporated into similar numbers of stromal cells was 3.6 to 1 or greater. In 13 samples (Table 2) satisfying the above criteria,
7 drugs were tested (Adriamycin in 12 samples, 1-β-D-arabino-
ofuranosylcytosine in one sample, daunomycin in one sam-
ple, 5-fluorouracil in one sample, melphalan in 2 samples, cis-
platinum in 3 samples, and vincristine in 3 samples) at several
concentrations. Where sample size permitted, 3 concentrations
per drug tested were chosen, ranging from 0.01 peak plasma
concentration to peak plasma concentration. Peak plasma
concentrations of drugs tested were taken from Alberts and
Chen (1). A total of 61 drug tests was evaluable using both
systems. For tumors where sample size permitted multiple-drug
testing, comparable drug sensitivity curves can be generated
for both the [H]thymidine incorporation assay and the HTSCA
as shown in Chart 3. A log scale was used for the [H]thymidine
coordinate, and a linear scale was used for the HTSCA coor-
dinate. In an ovarian ascites specimen, resistance to Adri-
amycin is demonstrated in both systems, whereas low dose
resistance and high dose sensitivity to 5-fluorouracil are dis-
played in both systems. The dashed line in the HTSCA panel
represents 30% of the mean number of colonies in the control
plates. Less than 30% colony survival has been used by von
Hoff et al. (8) to indicate drug sensitivity. The dashed line in
the [H]thymidine panel of Chart 3 represents 40% of control
[H]thymidine incorporation, which corresponds to the 30%
colony survival number used in the HTSCA as determined by
least-squares analysis of the 61 drug tests evaluated.

Using the definitions of sensitivity defined above, we then
classified 61 drugs tests as sensitive or resistant as shown in
Table 3. In comparing [H]thymidine incorporation to the
HTSCA, there is 89% concordance, i.e., 20 of the 61 deter-
minations are sensitive in both systems, and 34 of the 61
determinations are resistant in both systems (54 of 61 = 89%).
Of the 22 drugs tested at more than one concentration, 21
(95%) show either resistance in both systems at all doses
tested or sensitivity in both systems for at least one drug dose
determination.

**DISCUSSION**

Attempts to use radiolabel incorporation techniques for mea-
suring cell growth in soft agar systems have been limited in the
past by an inability to extract cells from the agar and variable
quenching of radioactivity that results from the presence of the
agar. Observations that decreasing percentages of agar re-
sulted in increased cloning efficiencies with P388 and L1210
cell lines, as well as our own observations that decreasing
percentages of agar also allowed increased cloning efficiency
in the HTSCA, led to consideration of a liquid top layer system.
This system incorporates the advantage of anchorage-depend-
ent cell growth inhibition in agar systems with the convenience
that a liquid culture system affords in terms of cell extraction
and [H]thymidine incorporation measurements.

A similar system has been reported by Yuhas et al. (10),
using 0.5% agar base and liquid top layer. These investigators
demonstrated visually that, in 11 transformed cell lines (8
mouse, 2 hamster, and one human), multicellular tumor spher-
oids formed and grew, whereas in 8 normal cell lines (6 mouse
and 2 human), cellular aggregates broke apart within 72 hr,
and the surviving cells failed to reestablish monolayer growth
when replated. These results are consistent with our findings
that normal human cells fail to grow or significantly incorporate
[H]thymidine in the liquid top system.

Optical counting experiments in the present liquid top sys-
tem, while demonstrating increased numbers of colonies per
plate compared with the HTSCA, still showed significant ob-
server variation in counting, and of course, the possibilities of
cellular reaggregation were increased, further adding to ob-

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* K. W. Kohn, personal communication.
server error. However, the ability to wash greater than 99.9% of the cells plated free of the agar base allows for ready utilization of a radioisotope incorporation method. We have shown that $[^3\text{H}]$thymidine incorporation is much greater in tumor cells than in nonmalignant cells. Day 4 was chosen to pulse the cells with $[^3\text{H}]$thymidine to allow a period of unperturbed tumor cell growth and to increase the difference in growth rates between tumor cells and anchorage-dependent normal cells, the normal cells being inhibited by the agar layer below, as Yuhas et al. (10) also observed. Cold TCA precipitation allows measurement of $[^3\text{H}]$thymidine incorporation into high-molecular-weight DNA from the non-agar-inhibited, i.e., tumor, cells. Twenty-four-hr $[^3\text{H}]$thymidine incubation was found to be optimal to maximize radioisotope incorporation into TCA-precipitable material.

Comparison of the present method to the HTSAC shows that they have several common properties, indicating that similar populations of cells are being measured in both systems. The number of viable cells plated is directly proportional to both $[^3\text{H}]$thymidine incorporation and the HTSAC colony count per plate. There is a strong positive correlation between drug dose-response curves generated in both assays. Overall, there is an 89% agreement in defining sensitivity or resistance for any drug dose tested. Twenty-one of 22 (95%) separate drug curves generated showed concordance between the 2 systems. In the experiment where discordant curves were obtained, results were close to the limits defining drug sensitivity, and if experimental error ranges were taken into account, they were compatible. Thus, the liquid top layer system is as likely to predict tumor sensitivity to a given agent, at any dose tested, as the HTSAC.

The liquid top layer system we have described incorporates properties of the HTSAC and has certain additional advantages in terms of in vitro drug testing. Since the underlayer is the same as that used for the HTSAC and the upper layer utilizes similar medium, the system presented here shares the properties of inhibiting growth of anchorage-dependent normal elements in tumor biology specimens and supporting the growth of a subset of tumor cells which has similar characteristics, in terms of drug sensitivity, to the subset grown in the HTSAC. The system presented here has the advantages of yielding results more rapidly, using smaller numbers of cells per sample, having smaller errors associated with the measurements, and using standard radiotracer incorporation techniques for the final measurements rather than more cumbersome and labor-intensive optical methods. Since the time required for the measurements of drug sensitivity is approximately 5 days, compared to 2 to 3 weeks for the HTSAC, the clinical utility of the result is enhanced, as a shorter time frame brings the test more into consonance with clinical staging times for cancer patients. It is still not ideal; a shorter time would be even more useful. Observer variation and questions of reaggregation of tumor cells are obviated, as label incorporation into high-molecular-weight DNA permits a direct objective measurement of the cells growing in the system.

The disadvantages of the present method relate mainly to the fact that the growing tumor cells are not relatively fixed in the upper layer as they are in the HTSAC. The assessment of individual colonies (or clones) is therefore not possible. The lack of fixation of cellular aggregates in the medium makes the clonal origin of these aggregates less certain than in the HTSAC and makes this system less suitable for studies related to cellular properties. However, these defects are not significant in terms of assessing drug sensitivities, although they would limit studies related to cell to cell variation within tumor specimens.

### Table 3: $[^3\text{H}]$Thymidine incorporation versus double agar stem cell assay

<table>
<thead>
<tr>
<th>Tumor</th>
<th>Drug</th>
<th>$[^3\text{H}]$Thymidine assay (% of control)</th>
<th>Double agar assay (% of control)</th>
</tr>
</thead>
<tbody>
<tr>
<td>Neuroblastoma cell line (CHP120)</td>
<td>Adriamycin</td>
<td>1.6 (S) 5 (S)</td>
<td>0 (S)</td>
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<tr>
<td>Ovarian ascites</td>
<td>Adriamycin</td>
<td>1.0 (R) 150 (R)</td>
<td>133 (R)</td>
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<tr>
<td>5-Fluorouracil</td>
<td></td>
<td>0.1 (S) 108 (S)</td>
<td>125 (S)</td>
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<td>Synovial cell sarcoma</td>
<td>Adriamycin</td>
<td>1.0 (S) 74 (S)</td>
<td>56 (S)</td>
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<td>Osteogenic sarcoma</td>
<td>Adriamycin</td>
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<td>61 (S)</td>
</tr>
<tr>
<td>Osteogenic sarcoma effusion</td>
<td>Adriamycin</td>
<td>0.6 (R) 58 (R)</td>
<td>38 (R)</td>
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<tr>
<td>Melphalan</td>
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<td>0.006 (S) 86 (S)</td>
<td>111 (R)</td>
</tr>
<tr>
<td>cis-Platinum</td>
<td></td>
<td>0.037 (R) 6 (R)</td>
<td>2 (S)</td>
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<tr>
<td>Neuroblastoma cell lines (KANR-27)</td>
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<td>36 (R)</td>
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<td>cis-Platinum</td>
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<td>0.006 (S) 115 (S)</td>
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<td>0.037 (R) 5 (R)</td>
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<td>Osteogenic sarcoma effusion</td>
<td>ara-C</td>
<td>1.0 (R) 44 (R)</td>
<td>26 (S)</td>
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<td>Adriamycin</td>
<td>0.6 (R) 46 (R)</td>
<td>5 (S)</td>
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<td>Melphalan</td>
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<td>Leiomysarcoma</td>
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<td>0.075 (R) 104 (R)</td>
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<td>123 (R)</td>
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<tr>
<td>Vincristine</td>
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<td>0.037 (R) 177 (R)</td>
<td>102 (R)</td>
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<td>Liposarcoma</td>
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<td>0.6 (R) 91 (R)</td>
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<tr>
<td>cis-Platinum</td>
<td></td>
<td>0.006 (R) 98 (R)</td>
<td>88 (R)</td>
</tr>
</tbody>
</table>

* S, sensitive; R, resistant (sensitivity being defined in the liquid top layer assay as less than 40% $[^3\text{H}]$thymidine incorporation in drug versus control plates and in the HTSAC as less than 30% colonies in drug versus control plates); ara-C, 1,β-e-arabinofuranosylcytosine.
Methodologies now available for assessment of in vitro drug sensitivity show great promise for both assessing the clinical utility of anticancer drugs and basic research on malignant cells. The liquid top layer system presented offers a method for independently evaluating in vitro drug sensitivity. Results appear to correlate with those of the HTSCA but are more rapidly achieved, use readily available laboratory technology, and have smaller variances. Further consideration of the liquid top layer system appears warranted.

ACKNOWLEDGMENTS

The authors wish to acknowledge Dr. Robert Ozols, Dr. Timothy Triche, Dr. Kurt Kohn, Dr. Avner Ramu, and Gloria Johnson for their advice and assistance in this project.

REFERENCES


Fig. 1. Synovial cell sarcoma primary explant after 3 days growth with 5 × 10⁵ cells plated per dish in the HTSCA (a) and liquid top layer assay (b). In the HTSCA, only one colony was seen on the plate. In the liquid top layer, there are larger and more numerous floating colonies. × 50.

Fig. 2. Human melanoma primary explant showing growth 10 days after 5 × 10⁵ cells plated per dish in the HTSCA (a) and liquid top layer assay (b). In the HTSCA, there are fewer clusters of cells, and no cluster exceeds 10 cells in number. In the liquid top layer, there are larger, more numerous colonies with pigmentation. × 20.
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