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# A Rapid *in Vitro* Labeling Index Method for Predicting Response of Human Solid Tumors to Chemotherapy[1]

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## SUMMARY

A rapid autoradiographic technique for measuring the [$^3$H]thymidine-labeling index of human solid tumors has been adapted to assess the effect of anticancer drugs *in vitro*. The drugs tested were present unchanged or as metabolites in serum obtained from patients immediately post-treatment. In 15 patients, the drugs tested *in vitro* were also given *in vivo*. Tumor-labeling index fell significantly in 5 of 6 patients who were later found to have objective clinical response. Tumor-labeling index did not change significantly in 8 patients and rose significantly in 1 of 9 patients who lacked clinical response. If confirmed, this *in vitro* test may prove to be a useful method of predicting responsiveness of human solid cancers to chemotherapeutic agents.

## INTRODUCTION

There is great interest in the use of human solid tumor systems instead of animal tumors in the screening of anticancer drugs and prediction of clinical response. One major system involves the assessment of drug effects on cells made to proliferate either in *in vitro* cultures (7, 11) or as heterotransplants to immunodeficient animals (1). The changes induced are usually determined morphologically, although quantitative methods are also available (13). Another widely used human-derived system is that of fresh or noncultivated tumor cells, studied as minces or suspensions in nutrient medium. Drug effects are measured qualitatively, *e.g.*, decolorization of methylene blue by succinic dehydrogenase (5), or quantitatively, by the incorporation of tracer materials into the tumors (2, 9, 16).

Two technical drawbacks of procedures requiring the cultivation of cells have been the variable success in achieving cell growth and the relatively long period of time needed to obtain results. For instance, even with the short-term cultures, using morphological criteria, a minumum of 3 to 4 days is required to assay drug effect.

Livingston *et al*. (12) have recently described a rapid and reliable autoradiographic technique for determining [$^3$H]thymidine LI[3] in fresh solid tumor cell suspensions. Results can be provided in 24 hr. We have adapted this method to study the action of anticancer drugs on tumor LI *in vitro*, with a view to predicting the subsequent clinical response to the drugs tested. We have tried to mimic the physiological environment of the cancer cell *in vivo*, at least in regard to drug concentration and state. Thus, instead of adding the drugs directly into the *in vitro* system (4, 9), we have tested the effect of drugs or their active metabolites as contained in serum, obtained from patients immediately after i.v. chemotherapy. Such a method has precedent in animal systems (8).

As might be expected, it has not always been possible to coordinate the laboratory testing of a particular specimen of tumor with the drugs given clinically, nor have we attempted to influence the clinical choice of drugs by the results observed *in vitro*. Nevertheless, we wish to report here our technique and the data thus far derived from 15 patients in whom a comparison between *in vitro* testing and clinical result is possible.

## MATERIALS AND METHODS

From 42 patients, 46 specimens were obtained by excisional biopsy of 25 superficial tumor nodules or by aspiration of 21 neoplastic effusions. Informed consent was given in all cases. Various neoplasms were represented: malignant melanoma (20 cases), breast adenocarcinomas (9 cases), other adenocarcinomas (7 cases), and miscellaneous nonadenocarcinomas (6 cases). All patients were in an advanced stage, with biopsy- or aspiration-accessible tumor, and were either previously untreated or were within 1 or 2 days before initiation of a new regimen of chemotherapy.

The drugs tested were obtained indirectly as follows: 10 ml of clotted blood were collected 5 to 10 min after the completion of i.v. administration of a drug or combination of drugs to patients, either the same patients (17) that provided the tumor specimens, or volunteer patients, not necessarily with the same diagnoses, but receiving the same drugs that were to be given to the patients we studied. In 6 subjects receiving p.o. and i.v. drugs, blood was drawn 2 hr after ingestion of medication and coincided with the 5th to 10th min after i.v. therapy. The so-called treated serum

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[3] The abbreviation used is: LI, labeling index.

thus derived was used for incubation of the tumor samples. A total of 18 different drugs, alone or in combination, were studied in the various tumor samples. Untreated or control serum was provided in 17 cases by the patients from which the tumor specimens had been obtained and, in the remainder, by normal healthy subjects.

Following the method of Livingston *et al.* (12), single cell suspensions of tumor nodules were prepared by mincing with scissors and forceps over a steel grid, and cells in effusion were concentrated by centrifugation. The cells were suspended in Ham's enriched medium containing 20% fetal calf serum or were left in effusion fluid in the case of neoplastic effusions. Tumor cell viability was determined by the trypan blue dye exclusion method.

Each sample of cell suspension was then split into 2 to 6 equal parts of 2 to 3 ml, with a cell count of 2 to 3 $\times$ $10^6$/ml, as determined by the Model B Coulter counter. One part was incubated immediately at 37° for 1 hr with [$^3$H]thymidine of high specific activity (6 Ci/mmole), using 5 $\mu$Ci/ml cell suspension. The 2nd part was placed in an Owen's bottle and kept in an enriched carbon dioxide (5%) incubator at 37° for 23 hr. Any remaining parts of the original sample were centrifuged, and the supernatant was removed. One cell pellet was resuspended in 2 to 3 ml of control serum, and each of the remaining pellets was resuspended in 2 to 3 ml of different treated sera. Each of these serum suspensions was similarly placed in an Owen's bottle and a carbon dioxide incubator at 37° for 23 hr. At the end of this time, each Owen's bottle cell suspension was transferred to an Erlenmeyer flask for the addition of [$^3$H]thymidine and the final hr of incubation. Just before this addition, a repeat trypan blue viability assay and cell count were performed. To prevent bacterial growth, 0.01 ml of kanamycin sulfate solution (100 $\mu$g) was added to each volume of cell suspension before prolonged incubation was started.

Incorporation of [$^3$H]thymidine was terminated by cooling on ice for 10 min. The cell suspensions were then layered on a Hypaque-Ficoll mixture (specific gravity, 1.080). The Hypaque-Ficoll gradient system was next centrifuged in the cold (4°) at 2500 rpm for 15 min. The buffy layer containing most of the viable cells was separated from the other layers, and 6 to 8 cytocentrifuge slide preparations were made from it. Two cytocentrifuge preparations were also made from the sediment. The slides were air-dried, fixed in 5% trichloroacetic acid and methanol, dipped in Kodak NTB-2 emulsion, exposed for 24 hr, developed, and finally stained with May-Grünwald-Giemsa. Fontana melanin stains were also done, in cases of melanoma. The autoradiographs were available for reading 48 to 72 hr after processing of a specimen was started.

The LI (the number of labeled cells per 100 tumor cells counted) was determined from a count of 200 to 1000 tumor cells (average 600) in 3 to 8 slides prepared from the buffy layer. A 2-fold change in LI values was considered significant ($p < 0.05$), based on statistical analysis of observer variability with this technique using split-sample fractions, as discussed in previous papers from our laboratory (12, 14).

**Chemicals and Commerical Suppliers.** Chemicals were obtained from the following suppliers: [$^3$H]thymidine (specific activity, 6 Ci/mmole), Schwarz/Mann, Orangeburg, N. Y.; Hypaque, Winthrop Laboratories, New York, N. Y.; Ficoll (M.W., 400,000), Pharmacia Fine Chemicals, Piscataway, N. J.; trypan blue dye, Grand Island Biological Co., Santa Clara, Calif.; Ham's medium and fetal calf serum, Grand Island Biological Co.; $D_{19}$ developer, $NTB_2$ nuclear track emulsion, and general-purpose fixer, Eastman Kodak Co., Rochester, N. Y.; and May-Grünwald and Giemsa stains, Curtin Scientific Co., Houston, Texas.

## RESULTS

**Control Incubations.** Table 1 summarizes the data from 42 patients. In 38 patients, comparisons of 1- and 24-hr Ham's or effusion fluid incubations were made; 34 (89%) of these had no significant change in tumor LI after 24 hr *in vitro*. In 28 patients, comparison of 1-hr incubation in Ham's or effusion to 24-hr incubation in control serum showed no significant change in 24 cases (86%). Corresponding LI's within the 2 24-hr groups were not significantly different in any of the specimens.

The cell counts of all the tumor suspensions after 24 hr, 40% lower in average than counts after 1 hr incubation, were still at adequate levels (mean, 1.6 $\times$ $10^6$/ml; median, 1.2 $\times$ $10^6$/ml; range, 0.5 to 5.0 $\times$ $10^6$/ml) to process meaningfully. The trypan blue viabilities of cells from the buffy layer, after 1 hr of incubation, for all patients, had a mean of 88%, a median of 90%, and a range of 32 to 100%; the respective figures for 24-hr incubations were 90, 86, and 30 to 100%. Thus, to the extent that the trypan blue dye exclusion assesses cell viability, the numbers of "dead" cells in the buffy layers are small and similar, irrespective of the period or the medium of incubation.

In the light of these findings, it was apparent that neither the 24-hr incubation nor the source of nutrient (Ham's effusion or human serum) had a major effect on the tumor LI, *per se*. We felt, therefore, that we could incubate reliably with control serum in this manner, and any changes in tumor-proliferative activity (*i.e.*, LI) in corresponding incubations with treated serum could reasonably be attributed to the drugs present.

The other types of tumors referred to in Table 1 are as follows: Tumors 30 and 31, adenocarcinoma of pancreas; Tumors 32 and 33, undifferentiated carcinoma of unknown primary; Tumor 34, adenocarinoma of rectum; Tumor 35, adenocarinoma of stomach; Tumor 36, adenocarcinoma of lung; Tumors 37 and 38, oat cell carcinoma of lung; Tumors 39 to 41, anaplastic carcinoma of head and neck area; and Tumor 42, malignant lymphoma.

**Treated Serum Incubations.** With specimens from 28 patients, a total of 55 incubations, 24 hr each, with treated serum were done; that is, each specimen was studied with 1 to 3 different sera. Eleven of these incubations, from 9 patients (32%), resulted in a significant fall in tumor LI as compared to control. In terms of the tumor types, 3 of 4 breast adenocarcinomas, 5 of 17 melanomas, and 1 of 7 miscellaneous cancers had a significant decrease in LI after 24 hr of exposure to treated serum.

In 15 patients, the drugs tested *in vitro* were identical to those received clinically for the 1st time, and the patients lived long enough for evaluation of at least 1 cycle of

Table 1
*LI of tumor cell suspensions incubated 1 hr compared to those incubated 24 hr*

| Patient | Tumor LI (%) | | |
|---|---|---|---|
| | 1-hr incubations in Ham's medium or effusion | 24-hr incubation | |
| | | Ham's medium or effusion | Control serum |
| Breast adenocarcinoma | | | |
| 1 | 14.4 | 13.2 | ND[a] |
| 2 | 4.0 | 2.8 | ND |
| 3 | 12.0 | 11.2 | ND |
| 4 | 9.0 | 10.6 | ND |
| 5 | 8.1 | 5.0 | ND |
| 6 | 4.8 | 4.5 | 3.3 |
| 7 | 15.0 | 11.5 | 10.0 |
| 8 | 11.4 | 7.2 | 9.1 |
| 9 | 15.5 | 15.1 | 9.8 |
| Melanoma | | | |
| 10 | 0.8 | 0.1 | ND |
| 11 | 6.0 | 9.0 | ND |
| 12 | 6.9 | 3.5 | ND |
| 13 | 10.9 | 4.0[b] | 4.0[b] |
| 14 | 4.5 | 2.7 | 2.1 |
| $15_i$ | 16.4 | 15.0 | ND |
| $15_{ii}$ | 16.8 | 16.2 | 21.5 |
| $16_i$ | 3.6 | 4.0 | ND |
| $16_{ii}$ | 3.7 | 2.4 | 4.0 |
| 17 | 4.2 | 3.8 | 3.7 |
| 18 | 6.6 | 1.5[b] | 3.7 |
| 19 | 6.7 | 1.8[b] | 3.8 |
| $20_i$ | 2.3 | 1.7 | 1.8 |
| $20_{ii}$ | 1.5 | 1.0 | 1.0 |
| $20_{iii}$ | 3.1 | ND | 2.0 |
| 21 | 9.4 | 13.0 | 12.0 |
| 22 | 8.4 | 11.4 | 13.5 |
| 23 | 7.1 | 13.3 | 14.0 |
| 24 | 2.3 | 2.0 | 3.7 |
| 25 | 1.6 | 1.0 | 2.0 |
| 26 | 4.8 | 3.5 | 2.2 |
| 27 | 1.7 | 0.8 | 1.0 |
| 28 | 17.0 | 5.0[b] | 4.0[b] |
| 29 | 5.0 | ND | 1.5[b] |
| Other types[c] | | | |
| 30 | 5.6 | 3.0 | ND |
| 31 | 6.5 | ND | 2.8[b] |
| 32 | 8.0 | 5.0 | ND |
| 33 | 9.8 | ND | 7.5 |
| 34 | 31.2 | 16.3 | ND |
| 35 | 4.7 | 5.2 | 4.4 |
| 36 | 20.0 | 17.3 | 15.6 |
| 37 | 29.2 | 19.2 | 21.0 |
| 38 | 21.5 | 12.8 | 11.8 |
| 39 | 31.0 | 26.5 | ND |
| 40 | 10.5 | 6.0 | ND |
| 41 | 8.5 | ND | 8.8 |
| 42 | 15.8 | 18.4 | ND |

[a] ND, not done, while feasibility of 24-hr incubations were initially being investigated.
[b] Significant change, $p < 0.05$.
[c] See text.

therapy. For these patients, it was valid to examine the correlation between *in vitro* findings and clinical results.

Table 2 indicates those patients who had a significant decrement in tumor LI *in vitro* (Group 1). All of these patients had objective clinical responses within 3 weeks after initiation of chemotherapy. Four other subjects with a significant fall in tumor LI are not listed here because the drug involved *in vitro* (adriamycin, cyclophosphamide, or actinomycin D) was either not given *in vivo* or additional drugs were given in producing the clinical response (2 cases). Patients 8, 21, and 25 also received immunotherapy, namely, *Bacillus Calmette-Guérin,* transfer factor, or *Corynebacterium parvum*. However, it is unlikely that the early clinical responses seen were due to these agents.

Patients in Group 2 demonstrated no significant fall in tumor LI *in vitro*. As is evident from Table 3, 9 of 10 in this group did not respond clinically, and, in fact, their cancers continued to progress. Patient 22, who was her own source for control and treated serum, was the only subject whose condition improved, beginning in the 7th week after the start of therapy. She was also on immunotherapy, as were the other patients in this group, except for Patients 9, 37, and 41.

Of 9 other patients (not tabulated) with no significant fall in tumor LI, in whom a comparison of the *in vivo* drug effect and *in vitro* result is not valid, only 1 patient responded clinically. This patient received a 3-drug combination, but her tumor was only tested with 2 of the 3 drugs *in vitro*.

**Overall Correlations.** Table 4 combines the results for the 2 groups of patients. It is clear that there is a high degree of association between the effect of drugs on tumor LI *in vitro* and the effect of the same drugs on tumor response *in vivo*. Specifically, it would appear that, with this *in vitro* test system, in most cases a significant fall in tumor LI predicts for clinical response to the drugs tested, while no significant fall predicts for absence of clinical improvement.

## DISCUSSION

The number of cases that could be adequately evaluated in this study is not large. However, this laboratory technique appears to have predictive ability at least equal to other *in vitro* methods using cultured (7, 11) or nondividing cells (4, 10) in human tumor systems. The test provides results in 2 to 3 days and is technically straightforward. Furthermore, we have obviated some of the arguments against the direct addition of drugs *in vitro* (6) with the use of treated serum, possibly thereby mimicking the pharmacological state and concentration *in vivo* of the drugs tested. Supporting this presumption was the production of a significant fall in LI by cyclophosphamide, a chemical requiring activation in the liver for its effects, in 1 breast carcinoma specimen. Burns *et al*. (3) have also used treated serum in a study of cytosine arabinoside therapy in acute nonlymphocytic leukemia, with findings supporting a correlation between depression of the *in vitro* LI by the drugs and subsequent clinical remission.

It is apparent that certain antitumor agents (namely, methotrexate and adriamycin) are active in unchanged form, and their pharmacology is well-defined to the degree that realistic levels of the compounds can be added directly to tumor cell suspensions. In such situations, it would be interesting to add known concentrations of drug directly and to compare the effects obtained with the results of exposure to treated serum containing the same drugs.

With regard to the drugs that can usefully be screened by

Table 2

*Patients with significant ($p < 0.05$) fall in 24-hr in vitro tumor LI (Group 1)*

| Patient | Tumor: Type | Tumor: LI (%), control serum | Tumor: LI (%), treated serum | Drug(s) used both *in vitro* and *in vivo* | Clinical results |
|---|---|---|---|---|---|
| 8 | Breast adenocarcinoma | 9.0 | 3.0 | Ftorafur[a] + cyclophosphamide + adriamycin | Partial remission: 50% decrease in size of s.c. mass; subsidence of pleural effusion |
| 13 | Melanoma | 4.0 | 1.5 | Hydroxyurea, i.v., *in vivo* | Partial remission: more than 50% decrease in size of skin nodules |
| 21 | Melanoma | 12.0 | 3.2 | Imidazole carboxamide + L-phenylalanine mustard | Improvement: less than 50% decrease in size of skin nodules |
| 25 | Melanoma | 2.0 | 0.5 | Imidazole carboxamide + L-phenylalanine mustard | Partial remission: more than 50% decrease in size of skin nodules; shrinkage of visceral metastases |
| 36 | Lung adenocarcinoma | 15.6 | 7.0 | Baker's antifol[b] | Partial remission: disappearance of lung lesion, but no change in bone metastases on X-rays |

[a] Related to 5-fluorouracil.
[b] Triazine folate antagonist, NSC 139105.

Table 3

*Patients with no significant fall in 24-hr in vitro tumor LI (Group 2)*

| Patient | Tumor: Type | Tumor: LI (%) control serum | Tumor: LI (%) treated serum | Drug(s) used both *in vitro* and *in vivo* | Clinical results |
|---|---|---|---|---|---|
| 9 | Breast adenocarcinoma | 9.8 | 15.7 | Adriamycin | No response |
| 14 | Melanoma | 2.1 | 0.9 | Actinomycin D | No response |
| 17 | Melanoma | 3.7 | 3.6 | Imidazole carboxamide + methyl CCNU[a] | No response |
| 20 | Melanoma | 1.8 | 2.7 | Actinomycin D | No response |
| 22 | Melanoma | 13.5 | 20.4 | Imidazole carboxamide + L-phenylalanine mustard | Improvement: Less than 50% decrease in tumor size |
| 24 | Melanoma | 3.7 | 14.0[b] | Imidazole carboxamide + L-phenylalanine mustard | No response |
| 28 | Melanoma | 4.0 | 5.8 | Imidazole carboxamide + L-phenylalanine mustard | No response |
| 33 | Adenocarcinoma, primary uncertain | 7.5 | 6.4 | Ftorafur + Baker's antifol | No response |
| 37 | Oat cell cancer, lung | 21.0 | 14.5 | Adriamycin | No response |
| 41 | Anaplastic cancer, head and neck | 8.8 | 8.1 | Anguidine[c] | No response |

[a] Nitrosourea compound.
[b] Significant rise ($p < 0.05$), but none of the other values in this column are significantly different from control values.
[c] Phase 1 drug, NSC 141537.

Table 4
*Comparison of clinical results in patients with and without significant fall in tumor LI in vitro*

| Effect of drug(s) on LI[a] | Effect of drug(s) clinically[a] | | |
|---|---|---|---|
| | No. of responders | No. of nonresponders | Total no. of patients |
| Significant fall | 5 | 0 | 5 |
| No significant fall | 1 | 9 | 10 |

[a] Effect on LI *versus* effect clinically: $\chi^2 = 7.8$ (with Yates' correction); $p < 0.01$.

our method, it seems that both cycle-specific agents, *e.g.*, Baker's antifol and hydroxyurea, and non-cycle-specific compounds, *e.g.*, imidazole carboxamide and adriamycin, can produce significant decreases in the LI's of various solid tumors within 24 hr. Our 24-hr control studies demonstrate that, with few exceptions, such a period of incubation in nutrient medium or human serum does not, *per se*, significantly affect the LI of tumor cell suspensions, relative to 1-hr incubations. Presumably then, the fall in tumor LI *in vitro* reflects the inhibition by the drugs of DNA synthesis both directly and indirectly, as a result of effects on RNA and/or protein synthesis. It would, of course, be unrealistic to expect perfect correlation between the *in vitro* drug effects and the *in vivo* results. The various reasons for this have been eloquently summarized (6) and include variation in the blood supply to a cancer mass and host factors such as the immunological system.

Murphy *et al*. (14), in an investigation of 48 patients, have demonstrated that the serial determination of LI can be applied to predict response in human solid cancers. Thus, in accordance with previous observations by Sky-Peck (15), they have found that a fall in the LI of tumors sampled postchemotherapy, compared to pretherapy, correlates with objective clinical response and that no fall or an increase in LI is accompanied by failure of response. This combined "*in vivo-in vitro*" approach has likewise been developed for acute nonlymphocytic leukemia by Zitoun *et al*. (17), with similar conclusions. If the results of the pretreatment *in vitro* test herein described are further substantiated, it might provide the additional advantage of direct applicability in the initial selection of drugs for the treatment of cancer.

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