**In Vitro Effects on DNA Synthesis as a Predictor of Biological Effect from Chemotherapy**

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**ABSTRACT**

Cell suspensions of tumor or normal bone marrow were incubated *in vitro* with control media (supplemented Ham’s F-10 medium or autologous, pretreatment plasma) and with autologous, posttreatment plasma containing chemotherapeutic agents or their metabolites (treated plasma). Drug-specific perturbation of tumor cell DNA synthesis, as measured by the thymidine labeling index, occurred in treated plasma in 9 of 9 courses where antitumor effect was seen compared to 2 of 8 in which chemotherapy failed. Similar effects on myelocyte labeling index in normal marrow were observed in 14 of 20 courses associated with leukopenia and in 0 of 3 in which there was none. The labeling index was affected in 23 of 29 courses with associated biological effect (79%) versus 2 of 11 courses without biological effect (p = 0.0007). Measurement of changes in labeling index upon exposure to drug-containing plasma may become a useful guide to the choice of antineoplastic chemotherapy.

**INTRODUCTION**

There has been renewed interest in the use of *in vitro* systems for the prediction of response to chemotherapy especially since the report by Salmon et al. (13) of their results with a colony-forming assay system. Previous efforts to measure changes in the rate of DNA synthesis *in vitro* as an indicator of therapeutic efficacy have met with disappointing results for solid tumors (16) although the results in acute leukemia were more promising (3, 17). These *in vitro* techniques have involved short-term incubation with antineoplastic drugs (up to 24 hr) and immediate harvest for determination of the thymidine labeling index or scintillation counting.

Effective chemotherapy delivered *in vivo*, regardless of its primary mechanism of action, consistently produces marked and prolonged suppression of DNA synthesis in sensitive animal tumors with the maximum effect usually evident at 48 to 96 hr (2, 7, 15). At earlier time points, there is either no effect or even an increase in thymidine uptake (11). Such apparent stimulation may relate to blockade of de novo thymidine synthesis by drugs like 5-fluorouracil or methotrexate under conditions which allow for use of the "salvage" pathway (utilization of preformed thymidine). Data for both chemotherapy and radiation therapy in the clinical setting suggest that a delayed, prolonged suppression of DNA synthesis after chemotherapy or radiation therapy *in vivo* is also characteristic of sensitive human tumors and that little or no suppression is associated with failure to respond (4, 9). Based on these observations, we felt that perturbation of DNA synthesis resulting from appropriate drug exposure *in vitro* might be predictive of biological events provided that sufficient time was allowed in short-term culture (up to 96 hr) to allow for maximal *in vitro* effect to occur.

We chose to expose cells *in vitro* to drug-containing plasma from the same patient, obtained just after administration of chemotherapy, in an attempt to mimic the *in vivo* situation as much as possible with regard to the availability of active metabolites at therapeutic concentration. Because of non-drug-specific possible effects of plasma itself, autologous pretreatment plasma and synthetic growth medium were used as controls. Our intent was to compare the thymidine labeling index in control media to that in drug-containing plasma at various time points and to examine the relationship of effects observed *in vitro* to biological effects. In the case of tumor tissue, we hoped to show an association with tumor response; in that of normal bone marrow, we hoped to show an association with myelosuppression.

**MATERIALS AND METHODS**

From April 1977 to May 1979, patients with accessible tumor and/or bone marrow who were to receive chemotherapy for their neoplastic disease were entered into the study. Informed consent was obtained in every case. Just prior to administration of chemotherapy by i.v. injection, 10 ml of control plasma and appropriate tissue specimens were obtained from the patient. Tissue specimens included bone marrow in 31 instances (8 containing tumor cells and 23 containing normal cells), pleural effusion containing tumor cells in 4, and ascitic fluid containing tumor cells in 4. One patient had biopsy of a tumor nodule. Within 5 to 15 min after completion of chemotherapy administration by rapid infusion, 10 ml of drug-containing (treated) plasma were obtained from the patient. Samples of bone marrow were drawn in a syringe and washed with preservative-free heparin. For pleural effusion or ascitic fluid, 100 units of heparin per 100 ml were added to prevent clotting. The crude cell suspension was layered on Ficoll-Hypaque to remove dead cells, RBC, and tissue debris (10). A similar procedure was followed with the one nodule except that a cell suspension was first prepared by finely mincing the tissue, forcing it through a wire mesh grid and suspending it in synthetic medium. Theuffy layer at the interface of supernatant fluid and Ficoll-Hypaque was removed with Pasteur pipets, and equal fractions were placed in separate Erlenmeyer flasks containing, respectively, 3 ml of Ham’s F-10 medium plus 10% fetal calf serum (medium control), 50% control plasma added to Ham’s F-10 medium to a volume of 3 ml, and 50% treated plasma added to Ham’s F-10 medium to a volume of 3 ml. The cell number incubated in each fraction was approximately $1 \times 10^7$ (3 x...
10⁶ cells/ml). Ham’s medium and fetal calf serum were obtained from the Grand Island Biological Co. After 24 hr of exposure to each medium in a humidified 5% CO₂ incubator (37°), each sample fraction was washed once and placed in Ham’s F-10 medium plus 10% fetal calf serum with 1% penicillin and streptomycin added. Cells (1 to 2 x 10⁶) from each fraction thus resuspended in control medium were then subdivided into 2-ml aliquots for further incubation periods to 48, 72, or 96 hr (concentration of 0.5 to 1.0 x 10⁵/ml), in Falcon plastic T-flasks.

Initially, at 24 hr (when the cells were resuspended) and at the remaining 3 time points, aliquots were harvested for determination of the thymidine labeling index by a method previously described (8). The labeling index was expressed as a percentage with at least 200 cells counted per determination. If tumor cells were the denominator, they were visually identified under oil immersion. When normal bone marrow was assayed, morphologically identifiable myelocytes were the denominator. The May-Grunwald-Giemsa stain was used on all autoradiographs.

Fortsy courses of varied chemotherapy, usually with multiple agents, were assayed in vivo and in vitro (17 for antitumor effect and 23 for myelosuppression). Antitumor effect in vivo was defined as complete response (disappearance of all measurable tumor for at least 1 month) or partial response (reduction in measured tumor area(s) by at least 50% for 1 month or more or estimated reduction in nonmeasurable disease by at least 75% for 1 month or more). Myelosuppression in vivo was defined as a reduction in circulating neutrophils after chemotherapy from normal levels to <2000/c mm. An effect on the labeling index in vitro was defined as a labeling index value in treated plasma at a given time point of incubation which differed from plasma control or medium control values by a factor of ≥1.9 (increase or decrease). A change of 1.9-fold or greater was considered significant based on our earlier observations with the same method of a variance of 0.01 for log counts of split-sample halves (8). A ratio of ≥1.9 between 2 labeling index values thus implies with 95% confidence that the 2 are representative of kinetically different cell populations. We examined the relationship between biological effect in vivo (tumor response or myelosuppression) and an effect of treated plasma on the labeling index in vitro. For the comparison of various proportions and testing for statistical significance, Fisher’s exact test was used.

RESULTS

There was no relationship between effects on the labeling index after 24 hr of in vitro incubation and biological effect. Among courses associated with tumor response or improvement, 2 of 8 evaluable at 24 hr were characterized by a labeling index effect in treated plasma versus either control while 4 of 6 courses without response demonstrated an effect. Only 2 of 18 courses associated with myelosuppression showed an effect in vitro on myelocyte-labeling index at 24 hr compared to 1 of 3 without myelosuppression. Similarly, no relationship existed between the height of the initial tumor cell-labeling index and the likelihood of response (mean, 14.0 for responders versus 12.9 for nonresponders).

A relationship was observed at 48, 72, and 96 hr between in vitro and in vivo effects. The relationship between antitumor effects from chemotherapy and labeling index perturbation at these time points is detailed in Table 1. Of 9 courses in which there was a clinical complete or partial response to chemotherapy, all 9 had an associated effect on labeling index in treated plasma versus medium control at one or more time points. In contrast, only 2 of 8 courses without antitumor effect had an associated labeling index change. Of 20 courses also using a variety of chemotherapeutic regimens in which myelosuppression occurred, 14 had associated labeling index perturbation in treated plasma versus control medium at 48, 72, or 96 hr. This was not seen in the 3 courses where myelosuppression failed to occur (see Table 2). Overall, an effect on in vitro labeling index was seen in 23 of 29 courses with biological effect (tumor response or myelosuppression) versus 2 of 11 courses without biological effect. This difference in sample proportions is highly significant by Fisher’s exact test (p = 0.0007). When treated plasma determinations were compared to those in control plasma at each time point, the results were essentially the same (21 of 26 evaluable courses with biological effect showed an effect in vitro at 48, 72, or 96 hr versus 3 of 11 courses without biological effect). These differences again are highly significant (p = 0.003).

The observed effect on labeling index in cells exposed to treated plasma vs. control medium in vitro is correlated with in vitro effects (inhibition, stimulation, or none by criteria defined in text) on thymidine labeling index of tumor cells.
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Table 2
Treated plasma effects on myelocyte labeling index in bone marrow and associated myelosuppression

<table>
<thead>
<tr>
<th>Diagnosis</th>
<th>In vivo chemotherapy</th>
<th>Outcome</th>
<th>48 hr</th>
<th>72 hr</th>
<th>96 hr</th>
</tr>
</thead>
<tbody>
<tr>
<td>AML*</td>
<td>A, V, ara-C, P</td>
<td>Leukopenia</td>
<td>↓</td>
<td>↓</td>
<td>↓</td>
</tr>
<tr>
<td>Breast</td>
<td>A, CTX</td>
<td>Leukopenia</td>
<td>↓</td>
<td>↓</td>
<td>↓</td>
</tr>
<tr>
<td>Lung, oast cell</td>
<td>MTX, V, A, CTX</td>
<td>Leukopenia</td>
<td>N</td>
<td>N</td>
<td></td>
</tr>
<tr>
<td>Lymphoma</td>
<td>CTX, A, V, P</td>
<td>Leukopenia</td>
<td>↓</td>
<td>↓</td>
<td>↓</td>
</tr>
<tr>
<td>Lung, oast cell</td>
<td>CTX, V, MTX, 5-</td>
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<td>↑</td>
<td>↑</td>
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<td>N</td>
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</tr>
<tr>
<td>Teratoma</td>
<td>V, A, CTX</td>
<td>Leukopenia</td>
<td>↓</td>
<td>↓</td>
<td>↓</td>
</tr>
<tr>
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<td>MTX, V, A, CTX</td>
<td>Leukopenia</td>
<td>N</td>
<td>N</td>
<td></td>
</tr>
<tr>
<td>Lung, oast cell</td>
<td>V, MTX, VP-16</td>
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<td>N</td>
<td>N</td>
<td></td>
</tr>
<tr>
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<td>Colchicine</td>
<td>Leukopenia</td>
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<td>N</td>
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</tr>
<tr>
<td>Lymphoma</td>
<td>CTX, V, A, P</td>
<td>Leukopenia</td>
<td>↓</td>
<td>↓</td>
<td>↓</td>
</tr>
<tr>
<td>Lung, oast cell</td>
<td>V, A, CTX</td>
<td>Leukopenia</td>
<td>N</td>
<td>N</td>
<td></td>
</tr>
<tr>
<td>Lung, oast cell</td>
<td>V, MTX, VP-16</td>
<td>Leukopenia</td>
<td>N</td>
<td>N</td>
<td></td>
</tr>
<tr>
<td>Breast</td>
<td>MTX, V, CTX, A</td>
<td>Leukopenia</td>
<td>↑</td>
<td>↑</td>
<td>↑</td>
</tr>
<tr>
<td>Lung, oast cell</td>
<td>MTX, V, CTX, A</td>
<td>Leukopenia</td>
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<td>N</td>
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</tr>
<tr>
<td>Carcinoid</td>
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<td>Leukopenia</td>
<td>N</td>
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<tr>
<td>Lung, oast cell</td>
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<td>Lung, oast cell</td>
<td>V, CTX, VP-16</td>
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<tr>
<td>Breast</td>
<td>MTX, V, CTX, A</td>
<td>Leukopenia</td>
<td>↑</td>
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<td>↑</td>
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<tr>
<td>Prostate</td>
<td>Bleomycin, mito-C</td>
<td>No leukopenia</td>
<td>N</td>
<td>N</td>
<td>N</td>
</tr>
<tr>
<td>AML*</td>
<td>MGBG</td>
<td>No leukopenia</td>
<td>N</td>
<td>N</td>
<td>N</td>
</tr>
</tbody>
</table>

* AML, acute myelocytic leukemia; A, anthracycline; V, vincristine; ara-C, 1-β-o-arabinofuranosylcytosine; P, prednisone; ↑, significant increase (see text); CTX, cyclophosphamide; N, no change; MTX, methotrexate; 5-Fura, 5-fluorouracil; ↓, significant decrease (see text); VP-16, 4'-dimethyl-epipodophyllotoxir-β, ϑ-ethylidene glucoside; mito-C, mitomycin C; and MGBG, methylglyoxal-bisguanylylhydrazone.

treated plasma was more often depression than stimulation. Of the 9 courses with clinical antitumor effect, 5 had associated depression of labeling index in vitro, and 4 demonstrated an increase. Thirteen of 20 courses which produced myelosuppression were associated with a decrease in myelocyte-labeling index, and only 2 were associated with an increase. (One course was associated with an initial increase and subsequent decrease). Among 11 courses of therapy without biological effect (tumor cells assayed in 8 and normal myelocytes in 3), stimulation was seen once, and depression was observed once in treated plasma. Methotrexate and/or 5-fluorouracil, drugs which are known to inhibit the de novo pathway of thymidine synthesis and which might therefore be expected to produce an increase in exogenous thymidine uptake as an initial effect, were part of the treatment regimen in 6 of 18 courses associated with depression and 5 of 7 courses where an increase in labeling index was observed.

Of interest is the fact that one patient with small cell lung carcinoma and marrow involvement by tumor, whose chest X-ray and marrow demonstrated response after chemotherapy, simultaneously had an increase in tumor cell-labeling index and a decrease in myelocyte-labeling index when his pretreatment bone marrow was exposed in vitro to treated plasma. Both types of in vitro effect were associated with biological events as he also became severely leukopenic.

We next examined the possibility that control plasma might itself be responsible for in vitro effects on labeling index and that these might be associated with biological events. Table 3 summarizes the frequency with which in vitro effects were observed, relative to results in medium control, and compares that to the frequency of in vitro effects in treated plasma according to the observed outcome in vivo. For any available determination at 48, 72, or 96 hr, an effect was observed in plasma control in 7 of 25 courses with biological effect versus 22 of 29 for treated plasma. Using a more strict criterion for "effect," that it had to be observed at 2 or more of the 48-, 72-, and 96-hr time points, this occurred in vitro on exposure to control plasma in 2 of 23 (9%) courses with observed biological effect while for courses in which no biological effect occurred, a similar in vitro effect was noted in 2 of 10 courses (20%). This is not a statistically significant difference (p = 0.351). On the other hand, in vitro effect of treated plasma at 2 or more of the 3 time points was noted in 16 of 27 courses with observed biological effect (59%) versus a similar in vitro effect in only 1 of 11 courses without effect in vivo (9%). This difference is significant at p = 0.005.

DISCUSSION

Our results indicate that plasma obtained from a patient shortly after administration of chemotherapy will produce effects on tumor cell DNA synthesis (as measured by the labeling index) which predict for subsequent success or failure of treatment. Similarly, effects on the labeling index of myelocytes appear to predict for development of drug-related leukopenia. The overall frequency of "false-negative" results (no effect in vitro but in vivo effect observed) is 24%, but false negatives occurred more often in prediction of leukopenia (6/20) than in the prediction of antitumor effect (0/9). The observed frequency of "false-positive" results (effect in vitro but none in vivo) is 2 of 11 (18%), but larger numbers of observations in this category will be necessary to know the true frequency of false positives. Among all evaluable courses, the in vitro result demonstrated a positive correlation with clinical outcome in 78% of the observations.

Since drug-containing plasma produced effects versus pretreatment plasma as often as it did versus serum-supplemented synthetic medium (79 and 81%, respectively, of courses with associated biological effect), we believe that human plasma factors per se are unlikely to affect the test. This is an important
observation since it implies the potential for assessment of antitumor effectiveness utilizing heterologous, drug-containing plasma obtained from other patients. In this way, it may be possible to screen an individual’s tumor against several possible drugs or drug combinations for efficacy before a decision is made to commit him to a given treatment program. The advantages to the use of drug-containing plasma, rather than direct assay of drugs added at various concentrations to a cell suspension, include the following: (a) plasma contains pharmacologically achievable levels of active metabolites as well as the parent compounds, which makes it possible to test drugs like cyclophosphamide; (b) a combination of agents may be readily assayed in a single test; and (c) factors such as plasma protein binding are incorporated in the in vitro situation.

One can speculate as to why some cultures demonstrated an increase in labeling index on exposure to drug-containing plasma. This may relate to blockade of de novo thymidine synthesis with a resultant decrease in the available pool of intracellular thymidine in the presence of mechanisms for incorporation of exogenous thymidine which remain intact. As cited (11), there is precedent in sensitive animal tumor models for a pattern of stimulation followed by depression of thymidine labeling index on exposure to drug-containing plasma obtained from other patients. In this way, it may be possible to screen an individual’s tumor against several possible antitumor effectiveness utilizing heterologous, drug-containing plasma.

The tumor stem cell assay system also appears to have potential usefulness as a clinical predictor of response to chemotherapy. Basically, it involves growth of tumor colonies in a 2-layer agar system with determination of drug efficacy based on a decrease in the colony counts of treated versus control cultures (5, 13). A recent presentation of results with this system in 128 evaluable courses of therapy reported a false-positive rate of 38% and a false negative of 4% using a single cutoff value for sensitivity in terms of drug concentration and time (14).

Both the labeling index perturbation test described here and the stem cell assay system have advantages and disadvantages, and they may have important features in common. The labeling index test has the advantages of providing a result more quickly (1 week versus 2 to 4) which may be important in the clinical situation. It allows for morphological verification that the individual cells which make up the counted population are, in fact, tumor cells. It has considerably greater simplicity, requiring an average of 8 hr or less technician time per test. Finally, it is potentially applicable to essentially all histological types of tumors while at present a substantial fraction of solid tumors and almost all leukemic specimens cannot be satisfactorily grown in the stem cell assay system. Although this series was composed primarily of material from bone marrows or effusions, the technique is easily applied to minced specimens of solid tumor (8). On the other hand, the labeling index assay as we have used it does not provide media which give clonogenic tumor cells a specific growth advantage as the 2-layer agar system apparently does (5); cells which label may not be stem cells and therefore may not have biological relevance; and, in tumors with a low labeling index (<5%), the counting of very large numbers of cells would be required to have any confidence about the significance of an observed effect. For that reason, we have excluded such specimens from analysis although labeling indices in this range are common in solid tumors.

We believe that each assay may be measuring effects of drug exposure on similar subpopulations of cells which are critical to continued cell renewal, namely, the fraction actively involved in DNA synthesis. Work by Hamburger et al. (6) and by other investigators (1, 12) with thymidine suicide in the tumor stem cell system has shown that short-term exposure to lethal amounts of tritiated thymidine results in the death of a majority of colonies, suggesting that a large proportion of clonogenic cells are replicating DNA at any point in time.

Further experience will be necessary to judge the relative merits of these assay systems. A useful clinical test will probably require further modification and development. However, their independent demonstration of considerable predictive efficacy suggests that treatment based on individual tumor characteristics, rather than empiricism, may soon become possible.

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