Clinical Application of the Clonogenic Assay*

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ABSTRACT

Simple methods were developed for cloning human solid tumors; 68 percent of the tumors processed formed at least 30 colonies within two to four weeks. The accuracy of the clonogenic assay for predicting clinical response was determined in a prospective, correlative study. Eighty-four patients had objectively measurable disease and had at least one course of chemotherapy. Tumor types included melanoma (33), lung (12), colon (7), breast (7), stomach (4), ovarian (12), sarcoma (7), and hepatoma (2). For patients whose tumors were sensitive \textit{in vitro} to a particular drug, clinical response was seen in 21/25 cases (84 percent). Tumor resistance was found in 59 instances, and 54 patients (92 percent) had no clinical response to the same drugs. Associations between \textit{in vitro} chemosensitivities and clinical course were highly significant.

Introduction

Cancer chemotherapy has long been an empirical science. Although drugs for treating individual cancer patients are selected on the basis of past experience or physician intuition, many solid tumors fail to respond to chemotherapeutic treatment, and patients are often subjected to the needless toxicity of ineffective drugs. The development of an accurate predictive test of tumor sensitivity to anti-neoplastic agents that is analogous to bacterial culture for antibiotic sensitivity has long been a high priority of cancer research. Early attempts to develop \textit{in vitro} tests for chemosensitivity of anticancer drugs failed because correlations between \textit{in vitro} activity and clinical course of the individual patient were inconsistent. A simple and efficient technique is described for performing a clonogenic assay for chemosensitivity testing on the most common human solid tumors. This report also presents correlations between \textit{in vitro} sensitivity testing and clinical responses.

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Methods

Design of Study

Chemosensitivity assays were performed on tumors from patients with primary or metastatic tumors. Drugs tested in vitro included those that the patient’s oncologist indicated would likely be used clinically. All patients then received standard chemotherapy based on pre-existing protocols. The selection of chemotherapy based on results of the clonogenic assay was not in the design of this study, but chemosensitivity data were provided to physicians for their information. Responses to chemotherapy were assessed by the clinician and were reviewed independently. Subsequently, the results of chemosensitivity testing were compared with each patient’s clinical response.

Preparation of Tumors

Connective and adipose tissues and areas of necrotic tumor were removed from surgical specimens. Tumor was weighed and minced into pieces less than 2 mm in diameter in the presence of CEM medium* containing 15 percent heat-inactivated fetal calf serum (FCS).† The CEM growth medium was developed in our laboratory for the culture of human solid tumors.³ Tumors likely to be associated with bacteria or yeast, such as colorectal tumors or melanomas from the skin, were decontaminated with three to five washings in CEM containing 15 percent FCS, 200 U per ml of pencillin,† 200 μg per ml streptomycin† and 50 μg/ml Fungizone.† These tumors were then suspended in CEM containing FCS and antibiotics for two to four hours at 4°C before two more washings with this solution.

Enzymatic Dissociation of Solid Tumors

Ten to 20 ml of enzyme medium consisting of Hank’s balanced salt solution (HBSS)† containing 0.03 percent DNase (500 Kunitz units per ml) and 0.14 percent collagenase Type I were used for each g of tissue. The tumor fragments were stirred for 90 min at 37°C in the presence of 5 percent CO₂. After enzymatic digestion, the free cells were decanted through 12 layers of sterile gauze and centrifuged at 200 × g for 10 min. The supernatant was removed and the cells were resuspended in CEM containing 0.03 percent DNase. The cells were centrifuged again and the pellet was resuspended in 10 ml of supplemented CEM.

Preparation of Agar Plates

An underlayer of agar was prepared from supplemented CEM as follows: 16.5 ml of supplemented CEM were warmed to 50°C and added to 3.5 ml of 3 percent agar, also prewarmed to 50°C. The solution was rapidly mixed and 1 ml was dispersed to each 35 × 10 mm well of a cluster plate. The agar was allowed to set at room temperature. Viable tumor cells, 1.2 × 10⁶, were suspended in 1.0 ml of supplemented CEM at 37°C. After 2.0 ml of a CEM/agar mixture at 50°C was added, the cells were rapidly and evenly suspended. One ml of cell suspension was then added to each of three wells over the feeder layer, yielding 4 × 10⁶ viable tumor cells per well. The plates were then placed in a humidified incubator at 37% in the presence of 5 percent CO₂.

Colony Counting

Plates were examined by inverted microscope at 4 × and 10 × twice a week.

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* MA Bioproducts, Walkersville, MD
† Flow Laboratories, Rockville, MD
† Grand Island Biological Co., Grand Island, NY
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Small clusters of cells usually were noted within the first week and the number of colonies reached maximum within three weeks. Most assays were counted within 14 to 21 days on an automated colony counting system, the Omnicon FAS II.** Minimum colony diameter was 60 microns (approximately 20 cells per colony). The means and standard errors for each triplicate count and the percent inhibition of colony formation by anticancer drugs relative to control wells were calculated. Assays with less than a mean of 30 colonies for the control well were considered as no growth and were not evaluable for drug effects.

Histologic Characteristics

Permanent slides of the tumor colonies were prepared and stained with hematoxylin and eosin. Special techniques, such as periodic acid-Schiff base and silver stains, were used when appropriate. Slides were prepared from the upper agar layer as follows: 2 ml of HBSS were layered onto each well, and excess proteins were eluted for 15 minutes at 37°C. Excess HBSS was drawn off, and this step was repeated. Two ml of 4 percent formaldehyde were added over the agar, and the plates were allowed to set overnight at room temperature. The formaldehyde was removed after 18 hours, and the plates were submerged in distilled water. The agar layers usually separated after a few minutes; however, if they did not, the top agar layer was gently dissociated mechanically from the feeder layer with needles. The top layer was floated onto a glass slide, slowly dried in a humidified atmosphere overnight, and then stained. Slides were examined by a pathologist (AJC) to determine the cyto-logic and morphologic characteristics of the colony-forming cells.

** Colony Inhibition by Anti-cancer Drugs

Chemotherapeutic agents prepared from standard intravenous formulations included doxorubicin hydrochloride (Adriamycin), bleomycin sulfate, Carmustine, dacarbazine, Fluorouracil, methotrexate sodium, mitomycin, Melphalan, Cisplatin, and vinblastine sulfate. Since the alkylating agent cyclophosphamide (Cytoxan) is inactive in vitro, Melphalan was used as the standard alkylating agent for in vitro testing. Its effects were assumed to be similar to those of cyclophosphamide. Each drug was tested in triplicate at a concentration shown in table I. Drugs were added to the tumor cell suspension (upper agar layer) immediately before plating. Drugs were prepared in 60× stock solutions and 100 μl of drug was added per 1.2 × 10⁶ cells. For control wells, 100 μl of CEM were added to the tumor cell suspension. Six control wells were usually plated.

Results

Growth and Identification of Colonies

Over 1000 tumors were processed, and 68 percent produced at least 30 colonies in soft agar culture. The major tumor

TABLE I

Concentrations of Anti-neoplastic Drugs Used for In Vitro Chemosensitivity Testing

<table>
<thead>
<tr>
<th>Drug</th>
<th>Concentration mg/ml</th>
</tr>
</thead>
<tbody>
<tr>
<td>Doxorubicin</td>
<td>0.4</td>
</tr>
<tr>
<td>Bleomycin sulfate</td>
<td>2.0</td>
</tr>
<tr>
<td>Carmustine</td>
<td>2.0</td>
</tr>
<tr>
<td>Dacarbazine</td>
<td>10.0</td>
</tr>
<tr>
<td>Fluorouracil</td>
<td>10.0</td>
</tr>
<tr>
<td>Methotrexate sodium</td>
<td>4.0</td>
</tr>
<tr>
<td>Mitomycin</td>
<td>3.0</td>
</tr>
<tr>
<td>Melphalan</td>
<td>1.0</td>
</tr>
<tr>
<td>Cisplatin</td>
<td>2.0</td>
</tr>
<tr>
<td>Vinblastine sulfate</td>
<td>5.0</td>
</tr>
</tbody>
</table>

** Feature Analysis System, Bausch and Lomb, Rochester, NY
types included cancers of the breast (100 grew/140 plated; 71 percent), colon (104/175, 60 percent), stomach (35/52, 57 percent), lung (62/85, 76 percent), ovary (50/67, 75 percent), soft tissue and skeletal sarcomas (72/122, 60 percent), and melanomas (135/189, 71 percent).

To examine the morphology of the cells and to detect features characteristic of the various histologic types of tumors, colonies were fixed and stained with hematoxylin, eosin, and special stains as appropriate. The cytologic characteristics and functional activities of the cells permitted accurate identification of the histologic type of tumor in most instances. Cells of all examined clones showed nuclear pleomorphism, high nucleocytoplasmic ratios, nuclear prominence, aminocytosis and cytoplasmic basophilia, features consistent with malignant cells.

**Correlation Between in Vitro Chemosensitivity and Clinical Course**

In vitro sensitivity to a chemotherapeutic agent was defined as greater than 50 percent tumor colony inhibition. Less than 50 percent inhibition of tumor colonies was defined as in vitro resistance. A complete clinical response was defined as disappearance of all apparent disease for at least one month. Partial response was defined as a 50 percent or greater reduction in the size of all measurable disease for at least one month. Patients whose disease stabilized, but who had no objective evidence of tumor regression, were considered nonresponders.

Of 400 patients evaluated, 84 had objectively measurable disease and received at least one course of chemotherapy. The correlations between in vitro sensitivity and clinical response are summarized in table II. There were 25 tumors for which the clonogenic assay predicted sensitivity, and 21 of these 25 patients had evidence of clinical response. For 59 tumors resistant to the drug tested, 54 of these patients showed no response to chemotherapy. Thus, the clonogenic assay had an accuracy rate of 84 percent for prediction of sensitivity (21/25), and an accuracy rate of 92 percent for prediction of resistance (54/59).

**Discussion**

Using the clonogenic method, suspensions of cells derived from a variety of disaggregated human solid tumors were grown in a matrix of tissue culture medium and agar. The techniques were less complex than those reported by Hamburger and Salmon, yet produced greater yields of viable cells and higher cloning efficiencies from solid tumors. Several investigators have shown that in vitro responses of individual tumors to selected chemotherapeutic agents correlated well with the clinical responses of the corresponding patients following treatment with the same drugs. However, most previous studies were done in a retrospective manner. One recent study with ovarian cancer was designed as a prospective correlative trial, and results of chemosensitivity testing in 40 patients showed a 62 percent true positive and a 99 percent true negative accuracy of prediction.

The purpose of our study was to confirm the accuracy of the clonogenic assay for predicting drugs likely to be active in
clinical treatment. The tumors used in this study were melanomas, sarcomas, and carcinomas of the lung, ovary, breast, stomach, and liver. In this prospective, correlative design, the clonogenic assay had a true positive accuracy of 84 percent and a true negative accuracy of 92 percent.

Although *in vitro* chemosensitivity results and clinical responses correlated well, technical problems involving tissue disaggregation, culture conditions, and colony counting were encountered. High yields of viable tumor cells rarely were obtained from primary carcinomas of the breast or colon, or from fibrous and skeletal sarcomas. Moreover, some tumor types (sarcomas, colon and gastric carcinomas) failed to grow as well as others (melanomas, lung and ovarian carcinomas). An obvious need exists for improvements in tissue disaggregation techniques. Culture media and media supplements such as animal sera, hormones, and antibiotics must be optimized for each tumor type. Quantitation of colonies at the completion of an assay was awkward, time consuming, and somewhat subjective with microscopic counting. With the use of the Omnicon FAS II, agreement between machine counts and visual observations was good, and the reproducibility of machine counts was excellent. However, one questions whether the machine can distinguish between true tumor colonies and non-viable features (clumps of dead cells, blood cell clots, non-disaggregated tissue). Assays that measure only proliferating tumor cells are under development in our laboratory.\(^\text{10}\)

The clinical usefulness of the clonogenic assay may be limited because (1) 32 percent of the tumors failed to grow in soft agar culture, and (2) many tumors were resistant to all drugs tested. Technological limitations of the assay may be overcome by improvements in tissue-processing techniques and culture conditions, but drug resistance is a limitation of the state of the art of chemotherapy. The clonogenic assay is most accurate for predicting resistance, but patients certainly may benefit from "negative" results by being spared the needless toxicity of ineffective drugs. The clonogenic assay no doubt will play a role in the development of more effective anticancer agents. Its use for the rapid screening of new antineoplastic drugs may result in potentially active agents moving more quickly into clinical trials.

Accurate predictions of chemosensitivity have been obtained for patients with a variety of tumor types, including multiple myeloma,\(^\text{5}\) sarcomas,\(^\text{6}\) melanomas,\(^\text{7}\) and cancers of breast,\(^\text{11}\) ovary,\(^\text{1,9}\) lung,\(^\text{6,11}\) stomach,\(^\text{6}\) and colon.\(^\text{6,7}\) This assay may have great value for individualizing therapy for the patient with cancer and for alleviating the needless toxic reactions from drugs destined to be ineffective. Large randomized prospective studies will be necessary to determine the overall impact of this predictive test on the treatment of solid tumors.

**References**