Limitations of the Fast Green Assay for Chemosensitivity Testing in Human Lung Cancer*

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Selection of patients with lung cancer who are most likely to benefit from chemotherapeutic treatment would be a substantial step forward. Therefore, a prospective study in predictive chemosensitivity testing in vitro using the fast green assay (FGA) as developed by Weisenthal et al was carried out. Sixty-six pretreatment tumor specimens were obtained, the majority by means of bronchoscopy (n = 42). Due to an initially insufficient yield of tumor cells (n = 19), dead cells in control samples after four-day culture (n = 15), contamination (n = 7), and laboratory failure (n = 2), only 23 (34.8 percent) samples were successfully tested. In 14 of 36 patients, a comparison between in vitro and in vivo response was possible. Taking into account the number of failures, this number of successful assays does not allow for any conclusion regarding accuracy of the FGA. We conclude that the FGA has limited usefulness for in vitro chemosensitivity testing in patients with disseminated lung cancer in whom biopsy specimens were taken without major surgical investigations. Future directions for predictive testing in vitro are discussed. (Chest 1991; 100:1358-63)

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The chemotherapeutic management of human lung cancer requires careful balancing between potential benefits and predictable side effects. Especially in non-small-cell lung cancer (NSCLC) this is obvious, because most chemotherapy regimens will produce responses in only a modest proportion of patients. Moreover, the effect on survival, also in those patients who are responding to chemotherapy, is limited. As a consequence, the administration of cytotoxic drugs to patients suffering from this malignant neoplasm on a trial and error basis is questioned by several authors.

In small-cell lung cancer (SCLC), most patients show impressive responses to combination chemotherapy regimens. However, in the vast majority of patients, the tumor relapses, and not all patients will benefit from second-line treatment. Thus, selection of patients with inoperable lung cancer who are most likely to have any benefit of chemotherapeutic treatment would constitute a substantial step forward. Therefore, we performed a feasibility study for a prospective trial of predictive chemosensitivity testing on fresh tumor biopsy material in patients with lung cancer who were considered candidates for treatment with the drugs tested in the in vitro assay.

**Materials and Methods**

**Tumor Cell Preparation**

Fresh human lung cancer material was processed as described in a previous report. Briefly, fresh human tumor specimens were placed in RPMI 1640 medium and 10 percent fetal calf serum (FCS) with penicillin (125 U/ml), streptomycin (125 mg/L), and amphotericin B (1 mg/L). If necessary, the specimens were grossly minced with scalpels and scissors. Since it is unnecessary to obtain single cell suspensions for the dye exclusion assay, excessive trauma was avoided. The cell suspension was incubated with 0.1 percent collagenase and 0.003 percent DNase for at least one hour at 37°C. Cells from malignant effusions were pretreated with a buffer (8.29 g NH4Cl, 1.0 g KHCO3, 0.0373 g Na2-EDTA/L distilled water) to lyse red blood cells. Cells from bone marrow aspirates were collected in heparin-containing (5,000 U/L) tubes. The number of viable nucleated cells was quantified by means of trypan blue exclusion. Based on our previous experience, a minimum number of 4 x 10^6 viable cells was considered necessary to perform a control and three drug-treated cultures.

**Drugs**

Vincristine for infusion (Eli Lilly, Indianapolis, IN) was dissolved in phosphate-buffered saline solution (PBS) and kept at 4°C at 20 times the desired concentration for a maximum of two weeks. Carboplatin for infusion (Paraplatin, Bristol Myers, Weesp, The Netherlands) was dissolved in 5 percent glucose at a concentration of 10 mg/ml and kept at 4°C for a maximum of seven days.

**Drug Sensitivity Assay**

The fast green assay (FGA) as described by Weisenthal et al was used with some minor modifications. The cells were resuspended in fresh Dulbecco's modified Eagle's medium (DME/F12 medium (1:1) supplemented with 20 percent FCS with a minimum concentration of 10^6 cells per milliliter with 10 fixed chicken red blood cells as internal standard and incubated for one hour at 37°C with vincristine 0.5 mg/L, carboplatin 18.5 mg/L, and combination

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of these two drugs using the same concentrations. These concentrations were chosen as the product of drug concentration x time were about two times higher as clinically achievable. In contrast to other investigators who used in vitro drug concentrations that were a fraction of clinically achievable peak plasma concentrations, we believed that at these concentrations it should both be possible to predict for drug resistance as well as for sensitivity. Untreated controls were incubated with the solvent used instead of the drug. After incubation, cells were washed three times with PBS and resuspended in 2.5 ml of fresh medium (DME/F12, 20 percent FCS) in polypropylene tubes for a short-term culture period of four days under a humidified atmosphere of 95 percent air, 5 percent CO2, at 37°C. At day 4, cells were stained with 1 percent Fast Green for 10 minutes and sedimented onto microscope slides using a centrifuge (Cytospin) (550 rpm, 5 minutes) and counterstained with a modified hematoxylin-eosin technique. Under the microscope dead cells stain green and the fixed chicken red blood cells, which are oval and nucleated, stain predominantly green. Living cells retain their characteristic appearance with hematoxylin-eosin. The fixation of the internal standard of the chicken red blood cells was performed as described before. A successful assay was defined as an assay in which at least 100 viable and recognizable neoplastic cells were present on the control slides and in which a result could be obtained for all drug-treated samples. The ratio of living tumor cells over fixed chicken red blood cells was determined for each triplicate of centrifuge slide of drug-treated cells. Results are expressed as percentage of untreated controls. A 30 percent cell survival was used as the cutoff between "response" and "resistant in vitro."

RESULTS

In Vitro Chemosensitivity Testing

Sixty-six pretreatment tumor specimens were obtained. Table 1 shows the origin and histologic features of the specimens. Most were obtained by fiberoptic bronchoscopy (n = 20) or by rigid bronchoscopy (n = 22). In all patients, at least one biopsy specimen was taken for histopathologic diagnostic procedures and as many as possible were taken for predictive testing. Fifteen of the bronchoscopy specimens (13 SCLC) and eight of otherwise obtained specimens (all SCLC) were taken from previously treated patients. Table 2 shows the results of the FGA. Finally, 34.8 percent (n = 23) of the specimens were assessable for chemo-specific testing in vitro (23.8 percent of the bronchoscopy specimens; 54.2 percent of the other specimens). The advantage of rigid bronchoscopy over fiberoptic bronchoscopy consists of the potential of the former method to obtain larger biopsy specimens. This is immediately shown by the fact that failure to perform a FGA on fiberoptic bronchoscopy specimens due to an initial insufficient number of cells was encountered in 60 percent (n = 12) of specimens (n = 20). For the rigid bronchoscopy specimens, this number was 22.7 percent (n = 5). The median number of cells, viable and nonviable, obtained with fiberoptic bronchoscopy was 1.5 x 10^6 (range, 0 to 1.4 x 10^6), median viability 7 percent, and with rigid bronchoscopy 7.0 x 10^6 (range, 2.7 x 10^6 - 9 x 10^6), median viability 16 percent. As in our previous study, a high percentage of cell death in control cultures after four days was found (Table 2). In 26.2 percent (n = 11) of the bronchoscopy specimens, all cells in the control samples were dead after four days' culture, which made drug evaluation impossible in these specimens. The mean number of cells obtained from the other

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<th>Table 1—Origin and Histologic Features of the Tumor Specimens</th>
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<td><strong>No. of Specimens (No. of Successful Assays)</strong></td>
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<td>Rigid bronchoscopy</td>
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<td>Lymph node metastases</td>
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<th>Table 2—Results of Fast Green Assay of Tumor Specimens</th>
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<td><strong>Bronchoscopic Specimens</strong></td>
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<td>Assessable with FGA</td>
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<td>Failure of FGA due to</td>
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<td>Initial insufficient number of cells</td>
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<td>Contamination</td>
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<tr>
<td>After 4 days all cells dead in control culture</td>
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<td>Laboratory failure</td>
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FIGURE 1. Chemosensitivity for carboplatin (18.5 mg/L for one hour) and vincristine (0.5 mg/L for 1 h) measured with fast green assay (FGA) in human tumor specimens.

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specimens was $4.9 \times 10^4$ (range, $4.4 \times 10^4$ to $4.3 \times 10^7$) all with a viability above 10 percent (range, 10 to 96 percent). In this group, there were only two failures due to an initial insufficient number of cells. Three specimens were not assessable owing to infection and two owing to laboratory failure. In four specimens, all cells were dead after four days' culture. These figures are somewhat higher compared with our previous study, in which there were no failures due to an initially low number of cells or after the culture period in controls. Twenty-three specimens (seven NSCLC and 16 SCLC) of the total number of specimens obtained could be evaluated for chemosensitivity testing in vitro with the FGA.

Figure 1 shows the results of the FGA is assessable. Of the SCLC specimens (n = 16), seven (43.7 percent) had less than 30 percent survival of viable tumor cells vs internal standard in the FGA after incubation with the combination of carboplatin 18.5 mg/L and vincristine 0.5 mg/L (range, 16 to 89 percent) (Fig 1). For the individual drugs, the median figures were 75 percent survival (range, 6 to 132 percent) for carboplatin and 69.8 percent survival (range, 20 to 157 percent) for vincristine (not depicted). There was no evidence found of synergistic drug effects. None of the NSCLC specimens assessable in vitro (n = 7) had more than 70 percent reduction of viable tumor cells vs internal standard (Fig 1).

**Comparison of In Vivo with In Vitro Response**

At the time of this study, patients with lung cancer were treated with various chemotherapy trials in our institution. The results of these studies are published elsewhere. Nineteen of the 37 patients with SCLC of whom tumor biopsy specimens were obtained for in vitro chemosensitivity testing were treated with carboplatin (400 mg/sq m) and vincristine (2 mg days 1 and 8) every four weeks in vivo. Eight (42.1 percent) of these specimens were assessable for response. For a comparison between in vitro and in vivo response, see Table 3. Six specimens were considered resistant in vitro (>30 percent tumor cell survival in the FGA) and two specimens were sensitive in vitro (<30 percent survival in the FGA). These specimens were obtained from eight patients with SCLC of whom four showed a partial response (as defined by World Health Organization [WHO] criteria) after two courses of carboplatin and vincristine and four had no clinical response.

Seventeen patients with NSCLC were treated with carboplatin and vincristine in the same schedule as mentioned above. Of all these patients, tumor biopsy specimens were available for in vitro testing. Six specimens were assessable for response in vitro; none showed more than 70 percent reduction of viable cells vs internal standard. Of the five patients from whom these specimens originated, three had progressive disease after one course of carboplatin and vincristine chemotherapy and two patients had stable disease after two courses of this regimen.

Thus, ultimately, in 14 (38.9 percent) of 36 patients, a comparison between in vitro and in vivo treatment was possible. Taking into account the number of assay failures, the small number of successful in vitro assays does not allow for analysis in terms of sensitivity or specificity of the FGA in this study. Using Fisher's exact test, there was a significant difference ($p<0.05$) between the number of SCLC specimens (7 of 16) and NSCLC specimens (0 of 7) with less than 30 percent survival to carboplatin and vincristine treatment in vitro.

**DISCUSSION**

There have been many attempts to develop tests to predict responses to anticancer drugs during the past 40 years. Two important uses for such a test are evident. First, it would be invaluable for screening new compounds and drug regimens for potential use in treating patients with cancer. Second, with pretreatment testing of a patient's tumor against a wide range of agents, it might be possible to tailor a patient's treatment individually. Despite the many proposed tests, none has achieved widespread acceptance. Mattern and Volm reviewed the ideal prerequisites of such a test: it should be technically simple, fast, inexpensive, reproducible, and applicable for all tumors. In addition, the test should be capable of taking into account the various methods of drug action and be able to predict with reasonable accuracy the clinical results. In this study, the FGA developed by Weisenthal and coworkers was used. This assay meets some of the above-mentioned criteria for the "ideal" in vitro chemosensitivity assay. However, the limitations of the FGA in bronchogenic cancers to this end are demonstrated by this study. In lung cancer, bronchoscopy is often the easiest way to obtain tumor material. A major obstacle for successful in vitro testing with this material was the initially low yield of viable tumor cells. Second, in the majority of specimens left, poor survival of the neoplastic cells in control cultures was the most common reason for failure assays. These problems were recognized by Weisenthal et al when testing human lymphatic neoplasms in vitro. In our previous study of in vitro chemosensitivity testing of

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<td><strong>SCLC</strong></td>
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*Two biopsy specimens originated from one patient.
human bronchoscopy specimens, only 25 percent of the biopsy specimens could be successfully tested with the FGA. Failures were again due to the same reasons as mentioned above. Some of these difficulties may be related to technical aspects of the initial assay set-up and the assay conditions itself. The low viability of tumor specimens at the beginning of the culture period may be increased by centrifuging pleural effusions and low viability specimens over Ficoll-Hypaque, followed by repeated washing in complete medium containing serum to remove the Ficoll-Hypaque. By this method, lysing malignant effusions with ammonium chloride would no longer be necessary, and may improve tumor cell survival in culture. Second, the addition of antigenic cells, such as avian red blood cells, at the beginning of the culture period, may have provoked secretion of several cytokines by tumor-infiltrating lymphocytes and macrophages present in the tumor specimen. As Weisenthal and coworkers have pointed out, the release of interferon \( \alpha \), tumor necrosis factor, and other cytokines may have an adverse influence on survival of tumor cells in vitro.

Several authors have suggested that the ability of tumor cells to survive in vitro may be of negative prognostic importance. Thus, one might argue that the low number of successful assays might be a reflection of the low number of therapy-resistant patients included in this trial. Indeed, in a recent report by Stevenson et al., the successful attempt to grow cell lines of NSCLC specimens was an independent negative prognostic factor for patient survival. In contrast, the same group found no significant survival differences in a group of 68 patients with SCLC between those whose tumor cell specimens grew in culture vs the others. Also, as carboplatin and vincristine produce a 16 percent response rate in NSCLC, the number of assay failures (12/19) in this group is most probably due to the assay itself. In other than bronchoscopy specimens, assay failure due to initially low number of cells was encountered only twice and no living cells after four days’ culture in four cases. The 55 percent success rate in these specimens is lower than the 75 percent success rate with these specimens in our earlier study. The number of viable cells obtained (median \( 4.9 \times 10^3 \)) would have allowed for clonogenic assays as about 5 \( \times 10^4 \) per dish are needed. However, when data from six studies using lung cancer specimens in the clonogenic assay are pooled, they show about 60 percent successful clonogenic assays (>30 colonies per dish). The FGA is easier to perform and faster. In addition, several authors have shown that results obtained with the latter assay show good correlation with the clonogenic assay. Therefore, in situations where a sufficient number of cells are available for drug testing, the FGA may have advantages over the clonogenic assay.

In the study under discussion, the FGA was used in a prospective setting. Although the low number of successful assays does not allow for a conclusive statement in regard to the accuracy of this test, there was a significant difference between tumor cell survival of the NSCLC and SCLC specimens after in vitro exposure to carboplatin and vincristine (\( p<0.05 \), Fisher’s exact test). This may be a reflection of the clinical differences in drug resistance known between SCLC and NSCLC. In a prospective clinical trial in extensive-stage SCLC, Gazdar et al. using the same assay as in our study, found a good correlation between results of in vitro drug sensitivity testing and response to primary treatment. Also, patients who received second-line chemotherapy based on these results (“\( \text{in vitro} \) best regimen”) had a significantly higher response rate and a suggestion of better survival. However, due to clinical limitations and the author’s strategy to create cell lines before in vitro drug sensitivity testing, only 16 (20 percent)—including two ineligible patients—of 80 patients entered into this trial were finally tested and entered prospectively in a “decision-aiding” mode for selection of treatment of refractory SCLC. Weisenthal et al. communicated a prospective trial in lymphatic malignant neoplasms using the FGA. Their results showed significant correlations (\( p<0.0001 \)) with the clinical response obtained in 69 patients with the same drugs as used in vitro.

A way to circumvent some of the problems encountered in this study may be to investigate specific properties of tumor cells thought to be associated with drug resistance rather than looking at cell kill in vitro. Recently, in three reports it was shown that expression of neuroendocrine antigens on lung cancer cells correlate with survival and response to chemotherapy. Salmon et al. saw significantly fewer responses to doxorubicin in vitro in myeloma, lymphoma, and breast cancer specimens whose tumor cells expressed the P-glycoprotein. Refinement of molecular biologic techniques should make it possible to study in the future the cellular localization and quantitation of expression of genes and gene products related to resistance in individual cells in tumor specimens. Alternatively, assays such as anthracycline accumulation may help to predict whether resistance mechanisms are functionally present.

We may conclude that the FGA has limited usefulness in in vitro chemosensitivity testing of human bronchogenic cancer. Assay failures are largely due to low yield of tumor cells by bronchoscopy and the low number of tumor cells surviving four days in vitro. Although with the use of suprapharmacologic drug exposures it may be possible to make specific predictions for drug resistance, a reliable and accurate predictive test for in vitro drug sensitivity remains a
rather distant objective."

REFERENCES


