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Correlation of *in vitro* Drug Sensitivity Testing of Long-term Small Cell Lung Cancer Cell Lines with Response and Survival

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***In vitro* drug sensitivity testing (DST) of long-term cultures from small cell lung cancer (SCLC) tumours was correlated with response and survival after four cycles of etoposide and cisplatin. 27 cell lines from 25 patients were tested by the semi-automated MTT assay after a median culture of 29 months. The logs of the IC_{50} concentrations for etoposide and cisplatin were correlated with each other. For both drugs, median IC_{50} values of patients with partial or complete responses ("responders") were significantly lower (7–8 fold) than those of non-responders. When survival was plotted according to whether drug IC_{50} values were in the upper or lower halves, curves for etoposide were significantly different, but those of cisplatin were not. DST of the long-term cell lines by MTT assay was significantly correlated with the Weisenthal dye exclusion assay of earlier passages of the same cell lines. DST of long-term SCLC cultures can predict clinical response and, for etoposide, survival. Disease-oriented panels of carefully selected, continuous, human tumour cell lines can be used to screen new drugs.**
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INTRODUCTION

THE DEVELOPMENT of the human tumour cloning assay [1] stimulated interest in using *in vitro* drug sensitivity testing (DST) to select individual therapy. Despite many technical improvements, logistic problems remain [2] and few prospective studies have been done [3–5]. Because empirical regimens for second-line therapy have limited value in many solid tumours [2–4] new drugs are required for refractory cancers. A variety of *in vitro* and animal models have been used, singly or in combination, to screen potential agents. One approach, currently in use by the National Cancer Institute, has been the development of disease-oriented panels of continuous human cell lines [6], but because cell lines may not fully reflect the properties of the tumours from which they were derived and because cell line properties may alter after prolonged culture time, this concept requires validation. We have demonstrated that DST of short-term and medium-term cultures (median 37 days) derived from small cell lung cancer (SCLC) predicted response to therapy [5]. We describe here that DST of continuous, long-term cell cultures derived from the same cohort of patients is correlated with clinical response and survival.

PATIENTS AND METHODS

Protocol design and patients

All patients studied had pathologically documented, extensive stage SCLC [7] and had been entered into a protocol for selection of individualised chemotherapy on the basis of *in vitro* DST [5]. Tumour samples for laboratory studies were obtained from routine, pretherapy diagnostic or staging procedures. DST testing with the Weisenthal dye exclusion assay [8] was done as soon as sufficient tumour cells were available (mean culture time 37 days). At least one tumour-containing specimen was obtained from 75% of patients, and our success rate for establishing long-term cultures from tumour-containing specimens was 36% [5].

After initial staging, four 3-week cycles of etoposide and cisplatin were administered. Patients were randomised to receive either standard or high-dose regimens for the first two cycles; thereafter standard dose treatment was given. Patients were then restaged to assess response. Complete response (CR) was defined as lack of clinical, radiological or pathological evidence of residual tumour. Partial response (PR) was defined as a greater than 50% reduction in the sum of measurable or assessable tumour lesions. Subsequent therapy depended on tumour response and the availability of DST results [5]: patients received either the "best" three drugs selected *in vitro* or, empirically, vincristine, doxorubicin and cyclophosphamide.

Cell culture and DST with the MTT tetrazolium dye assay

Specimens were handled and cultured as described [9]. Cells were cultured either in RPMI 1640 supplemented with 10% fetal bovine serum or in HITES medium [10] supplemented with 2% serum. The *in vitro* sensitivities of the cell lines to etoposide and cisplatin were measured with the MTT tetrazolium dye assay as previously described [11–14]. Minor modifications included testing in HITES medium supplemented with 2% serum, and trypsin disaggregation of the cells 1 day before seeding into microwells. Cells were resuspended in growth medium and seeded into 96 well plates in 180 μl. The assay was standardised for individual cell lines by predetermining the optimal seeding densities [11,13]. After approximately 16 h incubation (to allow cells to recover from trypsinisation), 20 μl drug or saline was added to test and control wells, respectively. After exposure to drugs for 4 days, the remaining steps were as

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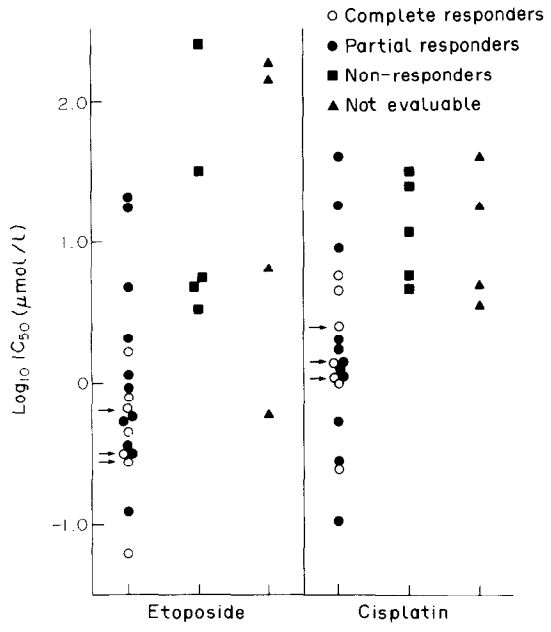

Fig. 1. Comparison of DST data with clinical response. Arrows = three cell lines from same patient.

described [11,13]. The results, reported as IC_{50} values were the means of three independent assays, each performed in eight replicates. IC_{50} values were the drug concentration required to decrease the optical density of the control sample by 50%. Because optical densities and cell concentrations are linear over a considerable range [15], IC_{50} approximates to the drug concentration required to inhibit cell growth by 50%. Personnel doing DST were blinded to the clinical data.

RESULTS

Cell lines

When these studies were done, 27 cell lines were available from 25 patients. 1 was derived from a lung biopsy specimen, and the others were from metastatic sites (bone marrow 10, lymph node 10, pleural effusion 2, liver 2 and subcutaneous masses 2). All cell lines grew as floating cell aggregates and had the morphological and biochemical features of "classic" SCLC lines [9,16].

Patients' responses

Of the 25 patients, 5 achieved a CR, 11 a PR and 5 had progressive disease or no response (NR) (Fig. 1 and Table 1). 4 patients died without tumour progression before restaging, and were considered to be not evaluable for response (NE). Whilst DST data from NE patients could not be used for correlation with response, they were used for correlation with survival. When tested, the cell lines had been in culture for a median of 29 months (range 5 to 46). Cell lines from three different metastatic sites were available from 1 patient (who achieved a CR). The Moses test (a non-parametric test of dispersion) applied to the logarithms of the IC_{50} values indicated that the three lines from the individual patient were significantly clustered compared with the other cell lines (two-tailed P values of 0.023 and 0.043 for etoposide and cisplatin, respectively). Thus, there were no significant differences in the drug sensitivity patterns of the lines from three metastatic sites. Because DST data from these lines could not be considered as independent observations, data from the line having the intermediate IC_{50} value for etoposide were arbitrarily selected for further correlations.

Table 1. DST data and survival

Response	Etoposide (μmol/l)*	Cisplatin (μmol/l)*	Survival (days)†	Culture time (mo)†
CR (n=5)	0.45 (0.06–1.70)	2.43 (0.25–5.5)	542 (456–921)	24 (13–42)
PR (n=11)	0.92 (0.12–20.5)	1.40 (0.11–46.5)	222 (107–990)	34 (17–43)
CR + PR (n=16)	0.69 (0.06–20.5)	1.55 (0.11–46.5)	340 (107–990)	31 (13–43)
NR (n=5)	5.4 (3.3–250)	11.5 (4.7–30.0)	176 (63–349)	33 (5–39)
NE (n=4)	74.8 (0.6–184)	11.4 (3.4–41.7)	13 (4–97)	18 (6–46)
All cases (n=25)	1.70 (0.06–250)	4.50 (0.11–46.5)	222 (4–990)	29 (5–46)

*Mean (range) of the $\log_{10}$ of IC_{50}.
†Median (range).

To determine whether the 25 patients were representative, we compared their survival (by the logrank test), and various combinations of the four types of clinical responses (by χ^2 analysis) with those of the other 62 patients entered onto the protocol. There were no significant differences between the two populations.

Correlation of DST data and clinical response

The responses of all 15 patients who were randomised to standard dose or high-dose arms were evaluable. 7 of 9 receiving a standard dose achieved a PR or CR (78%) compared with 4 of 6 receiving a high dose (67%) (not significant, Fisher's exact test). We have previously reported that there were no significant differences in response or survival between the two arms [17], and an update of all protocol patients confirmed this finding (response rates in both arms were approximately 80% and median survival of both groups was 11 months). Because of these findings, data from both regimens were combined for further analyses.

We compared the DST of the three response groups, CR, PR and NR (Table 2). An analysis of variance test for normality demonstrated that the logs of the IC_{50} values had a Gaussian distribution; therefore parametric tests could be used. Analysis of variance indicated that the three groups did not have equal means. For etoposide, pair-wise comparison of the three groups demonstrated that the NR group was significantly different from

Table 2. Correlation of DST with clinical response: statistical analyses

Comparison	Two-tailed P	
	Etoposide	Cisplatin
CR vs. PR vs. NR	0.0072	0.078
CR → PR → NR	0.0041	0.057
CR + PR vs. NR	0.0036	0.022

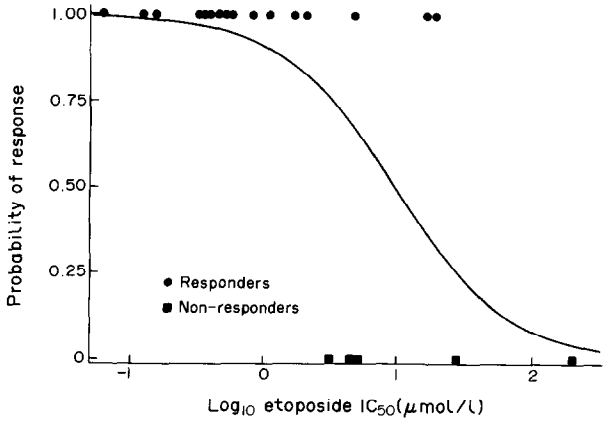

Fig. 2. Logistic regression model to estimate probability of response as explicit function of IC_{50} values for etoposide.

each of the other two (data not shown). However, for cisplatin, the NR group was only marginally different from the CR group, and not significantly different from the PR group. The CR and PR groups were not significantly different from each other. However, Jonckheere's test showed a significant trend from CR to PR to NR groups for both drugs (Table 2). When the data of the CR and PR groups were combined and compared with the NR group, the *t* test demonstrated significant differences between the two groups for both drugs (Table 2).

We used logistic regression to estimate the probability of response as an explicit function of the IC_{50} values for etoposide and cisplatin. The fit of the models for both drugs was significant (etoposide two-tailed $P = 0.0036$; cisplatin two-tailed $P = 0.004$). From the curve illustrated in Fig. 2, it could be estimated that the probability of response (CR or PR) was greater than 90% if the IC_{50} of etoposide was less than 1.2 μmol/l; the probability was about 50% if the IC_{50} was near 11 μmol/l; and the probability was less than 10% if the IC_{50} was greater than 99 μmol/l.

Correlation of DST data and survival

The patients were stratified by upper and lower halves of the drug IC_{50} values. Kaplan–Meier survival curves for etoposide were significantly different (Fig. 3), but those for cisplatin were not. Median survival was about 12 months in the group with the low values, and about 5 months in the group with high values. The overall median survival was 7 months.

Correlation of etoposide with cisplatin

Pearson product moment correlation demonstrated that the $\log_{10}$ IC_{50} values of the two drugs were strongly correlated (Fig. 4).

Correlation of DST results of newly cultured and established cell lines

The results obtained by the MTT assay (after a median culture time of 29 months) were correlated with those of earlier passages of the same cell lines with the Weisenthal dye exclusion assay (after a median culture time of 37 days) (Table 3).

DISCUSSION

There are several reasons why SCLC is an important model for *in vitro* DST studies. While most SCLC tumours in previously untreated patients are sensitive to chemotherapy, about 20–25% show *de novo* resistance [18,19]. Most tumours that respond to chemotherapy eventually recur, at which time they usually demonstrate resistance to multiple agents. Because most SCLC patients are not candidates for surgical resection of the primary tumour, laboratory samples usually contain small numbers of viable tumour cells obtained during routine diagnostic or staging procedures. In many cases, tumour cell numbers must be increased by *in vitro* culture before DST can be done. Cell lines can be initiated from more than one-third of patients from whom at least one tumour-containing sample reaches the laboratory [5]. Thus, representative cell line banks can be established. The ability to start a cell line from a tumour-containing specimen is not a major negative prognostic factor for extensive stage SCLC [20]. The metastatic sites from which the cell lines were derived included the common sites sampled during routine diagnostic and staging procedures. In 1 patient, cell lines were established from three metastatic sites. The DST results of these three lines were similar, suggesting that variations in the sensitivities of

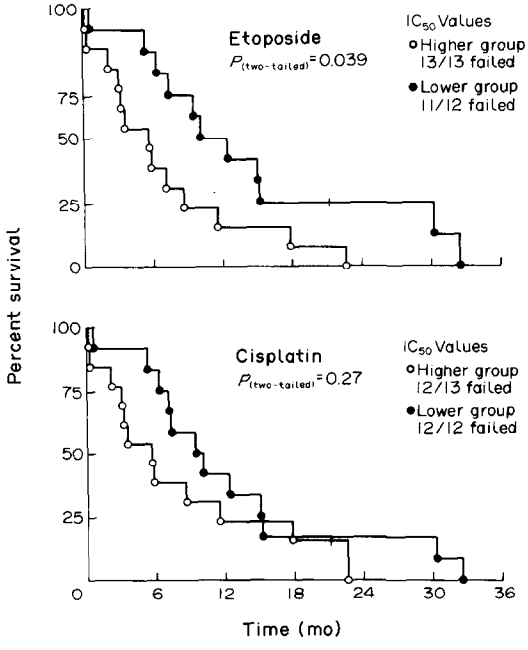

Fig. 3. Comparison of DST data with patients' survival by Kaplan–Meier curves. Patients were stratified by upper and lower halves of IC_{50}.

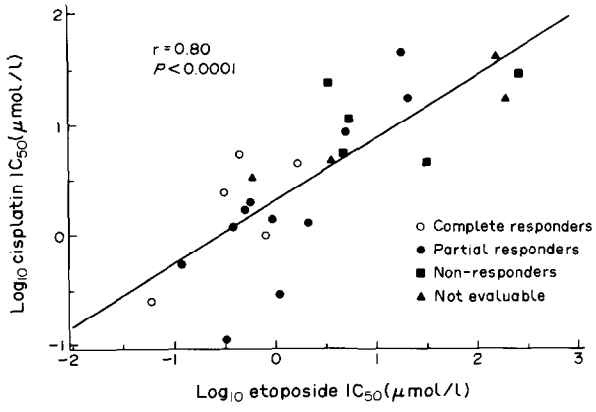

Fig. 4. Correlation of log IC_{50} values of etoposide and cisplatin.

*Table 3. Correlation of Weisenthal dye exclusion and MTT assays on 23 cell lines after short-term and long-term culture, respectively**

Drug	r	P
Etoposide (n=23)	0.69	<0.001
Cisplatin (n=18)	0.41	0.045

*Variable is IC_{50} for MTT assay and % cell survival for Weisenthal assay. r = Pearson product-moment coefficients and P is one-tailed.

individual metastatic sites may not present a major problem. These limited data are in contrast with the findings of Von Hoff *et al.* [21], who found considerable heterogeneity in the drug sensitivity profiles of different metastatic sites in individual patients with the clonogenic assay.

The MTT assay has been widely used for the *in vitro* drug sensitivity testing of several human tumour types including lung [6,11,14,22–24]. Because it does not discriminate between malignant and stromal cells, the application of the MTT assay to fresh human tumour samples is limited. In addition, for optimal results, the assay conditions have to be individualised for each cell line. Under these circumstances, the MTT assay yields similar results to clonogenic assays [11,14,24]. Using the MTT assay to test long-term established cell lines, we found a wide range of IC_{50} for the two drugs tested (about 4000 fold for etoposide and about 400 fold for cisplatin). Using a different set of lung cancer lines and slightly different assay conditions, Carmichael reported about a 250 range for etoposide and about 50 fold for cisplatin [12]. The high ranges may reflect an artefact of the testing system (which measures the drug concentration required to inactivate metabolically 50% of the cells after a 4-day exposure), or it may indicate that the variations in SCLC sensitivities are much greater than previously appreciated. Using an assay different from ours which measured clonogenic potential or thymidine uptake, Kern and Weisenthal [25] tested 450 human tumours of various types with suprapharmacological drug exposures (about 100 fold higher than other reported exposures). Even at these high drug concentrations, however, 18–42% of patients, depending on tumour histology, had tumours showing extreme drug resistance *in vitro*. Less than 1% of these patients responded to chemotherapy. Our results in previously untreated SCLC patients, with a quantitative assay, were similar—cell lines exhibited a wide range of IC_{50} concentrations, and DST results were correlated with clinical response.

Our data demonstrate that DST of continuous cell lines established from SCLC tumours can predict for response to therapy and survival. We confirmed that our lines had been established from a representative patient population with extensive stage SCLC. The data from etoposide and cisplatin were significantly correlated. However, DST results with etoposide were more predictive of clinical outcome than with cisplatin. While the response rates of both etoposide and carboplatin are high when administered as single agents in previously untreated SCLC patients, cisplatin has never been adequately tested in previously untreated patients [26]. Thus, the relative efficacies of etoposide and cisplatin as single agents in previously untreated SCLC patients cannot be compared.

We have previously demonstrated that DST of short-term and medium-term SCLC cultures predicts for response to therapy and survival. Our present studies were on cultures derived from patients entered onto the same protocol. However, there were several important differences between the two studies. Perhaps the most important was the current use of permanent cell lines that had been cultured for a median of 29 months before testing. Previously, we used the Weisenthal dye exclusion assay, which yields semi-quantitative data. The MTT assay is objective, semi-automated, less laborious, more reproducible, and determines exact IC_{50} values, permitting more precise correlation. The results of the two assays were correlated with each other, indicating that DST patterns of cultures are stable over lengthy culture times. Of interest, Alley *et al.* [6] also demonstrated that the DST profile of established cell lines, with the MTT assay, were stable over multiple passages over several months. The MTT assay is suitable for screening new antineoplastic agents [6]. The use of established cell lines allows repeated testing with multiple agents. Cell lines can be distributed to other laboratories for confirmatory and other studies, which could include investigation of mechanisms of drug resistance.

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Primary Hypothyroidism Associated with Interleukin-2 and Interferon Alpha-2 Therapy of Melanoma and Renal Carcinoma

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Four patients out of twenty with renal cancer and melanoma undergoing cancer immunotherapy with interleukin 2 (IL-2) and interferon alpha-2 (IFN-α_2) had laboratory evidence of hypothyroidism starting at cycle three to six, with a decline in serum thyroxine below normal and, in three cases, a rise in serum thyrotropin and thyroglobulin. One hypothyroid patient had elevated serum antimicrosomal antibody titres before the start of treatment and two others responded similarly during therapy. Three of the sixteen euthyroid patients also developed elevated titres of this antibody. Partial or complete remission was observed in seven of the patients—three of the four with hypothyroidism showed tumour regression. Thus IL-2 and IFN-α_2 can cause hypothyroidism, presumably via induction or exacerbation of autoimmune thyroid reactions. The occurrence of hypothyroidism may be mediated by high-dose IL-2 (rather than by LAK cell therapy as previously suggested) and potentiated by IFN-α_2.
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INTRODUCTION

INTERLEUKIN-2 (IL-2) stimulates the *in vitro* tumoricidal activity of peripheral blood mononuclear cells (PBMC) via induction of lymphokine-activated killer (LAK) cells, which are capable of lysing natural killer (NK) resistant tumour targets [1, 2]. Trials with IL-2 alone or with LAK cells have reported promising results in patients with advanced renal carcinoma and melanoma [3]. Previous studies reported little correlation between laboratory and clinical variables and the response to IL-2 and/or LAK cell therapy [4], except for clinical response and HLA-DR expression by tumour cells [5] and the occurrence of autoimmune hypothyroidism [6]. Autoimmune hypothyroidism was observed in a fifth of patients receiving high-dose IL-2 with LAK cells. Most of these hypothyroid patients had thyroid autoantibodies before therapy. Within 6–11 weeks after therapy, thyroxine (T4) levels declined with an inverse rise of thyroid-stimulating hormone (TSH); in some cases, clinical hypothyroidism was observed. In contrast, 11 melanoma patients receiving IL-2 alone by the same schedule showed no hypothyroidism. It was suggested that LAK cells are required for the development of hypothyroidism in these patients. Furthermore, tumour regression rate was higher in patients who developed hypothyroidism than in euthyroid patients (71% vs. 19%).

We report primary hypothyroidism in four (three responders and one non-responder) out of twenty renal cancer and melanoma patients undergoing immunotherapy with IL-2 and interferon alpha-2 (IFN-α_2). The results suggest that combined treatment with these cytokines, rather than LAK cell administration, is required for the development of hypothyroidism in these patients.

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PATIENTS AND METHODS

Study design

Patients were treated in the clinical oncology unit of the Istituto Regina Elena, Rome, as part of a multicentre trial with