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In vitro Prediction of Cytostatic Drug Resistance in Primary Cell Cultures of Solid Malignant Tumours

Manfred Dietel, Ute Bals, Birgit Schaefer, Ingrid Herzig, Hartmut Arps and Maciej Zabel

The *in vitro* monolayer proliferation assay (MP-assay) described here enables predictive determination of the efficacy of anticancer drugs considered for clinical application. The assay was designed (1) to achieve a high plating efficiency, (2) to adapt *in vitro* growth as close as possible to *in vivo* conditions, and (3) to prove that the cells *in vitro* correspond with the *in vivo* tumour cells they were derived from. From 452 freshly explanted or biopsied tumours, 321 (71%) proliferating cultures could be established. To prove malignant origin of the incubated cells each strain was characterised by DNA-cytophotometry for aneuploidy and by immunocytochemistry for marker proteins. Drug potency was determined by comparing the number of living cells in drug-treated cultures with non-treated controls. Drug concentrations *in vitro* corresponded with those achievable in tumour tissue and thus represented clinically relevant levels. Growth inhibition *in vitro* was correlated with *in vivo* tumour response. Two hundred *in vitro/in vivo* correlations were performed (50 retrospective, 150 prospective). Overall predictive accuracy of the MP-assay was 86%, with correct indication of resistance in 94.5% and of sensitivity in 75.8% ($P<0.001$). The results show that the proposed assay is capable of estimating the response probability of cytostatic drugs in individual tumours and thus can contribute to reducing the applications of non-effective drugs and, within limitations, to improving the basis of drug selection.
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INTRODUCTION

IN CHEMOTHERAPY of cancer patients it is often difficult to know which cytostatic drug will be most potent in reducing tumour growth or which will be inefficient due to tumour cell resistance. Usually the selection is deduced from empirical clinical studies based on a large number of patients treated under various conditions. However, for individual tumours this may have limited significance [1, 2]. Consequently, prior to chemotherapy it is often not known whether a patient will benefit from the drug(s) applied or whether toxic side-effects will dominate. Hence, a reliable test to predict drug effectiveness would be highly desirable [3]. Several assay systems have been developed to address this problem [for reviews see 4–8], of which only a few have reported acceptable plating efficiency, adequate time of testing, and satisfactory *in vivo/in vitro* correlations [3, 9–12].

Several reasons for the difficulties may exist [11], one of which is certainly the heterogeneity in chemosensitivity of different cell clones within a given lesion. However, the heterogeneity in resistance appears to be less marked, making prediction of chemoresistance more feasible. A second drawback to many *in vitro* assays may be the artificial nature of the test, e.g. tumour cells are taken from the patient, treated with the drug for example 1 h and only then cultured for growth. This procedure is unsuitable for simulating *in vivo* conditions.

The chemoresistance assay described here was designed to adapt *in vitro* test conditions as close as possible to the *in vivo* situation of cancer cells under chemotherapy. The major procedural points were: (1) prior to testing, the cells in culture underwent characterisation to prove their tissue specific origin by immunocytochemistry, e.g. keratin in carcinoma-derived cells; (2) to ensure malignancy, the nuclear DNA content was determined by cytophotometry in each culture; (3) effects of the drugs were determined in proliferating cultures by counting living cells (procedures using artificial "growth-related" criteria such as dye exclusion, tetrazolium colometry, etc. were avoided); (4) drug potency was evaluated at therapeutically achievable concentrations; (5) tumour cells were cultured in the presence of a small proportion (max. 10%) of normal stromal cells from the same tumour in order to more closely simulate physiology than, e.g. clonogenic growth in agar; and (6) each drug considered for application was tested separately.

The present study reports procedures for establishing cell cultures, cell characterisation, determining drug effects and for the correlation of *in vitro* results with *in vivo* outcome.

MATERIALS AND METHODS

Tumour material and culture technique

Explanted tumour tissue was sent to the laboratory immediately after surgery, avoiding cooling or other manipulations. A portion was processed for routine histology. The procedure for preparing primary cultures is described in detail elsewhere [13–15]. In brief, single cell suspensions or small tissue fragments were obtained by dissecting the material mechanically by scraping and/or by enzymatic digestion for approx. 20–30 min. with collagenase/dispase 0.1 U/ 0.8 U/ml (Boehringer, Mannheim). After centrifugation (600 rpm, 25°C) sediment was plated and incubated at 37° in 95% air and 5% CO_2. To prevent fibroblast overgrowth differential trypsinisation with brief exposure times was performed taking advantage of the different trypsin sensitivity of mesenchymal cells, or it was performed

Correspondence to M. Dietel.
M. Dietel, U. Bals, B. Schaefer and I. Herzig are at the Institute of Pathology, Christian-Albrechts-Universität zu Kiel, Michaelisstr. 11, D-2300 Kiel 1, F.R.G.; H. Arps is at the Institute of Pathology, University of Hamburg, F.R.G.; and M. Zabel is at the Medical Academy of Poznan, Poland.
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by mechanical removal of fibroblast colonies. For culturing, Leibovitz L15 medium was used, supplemented with 10% fetal calf serum (Gibco, Eggenstein, F.R.G.), 500 mg glutamin, 3.1 mg fetuin, 40 IE insulin, 1.25 mg transferrin, 0.56 g $NaHCO_2$, 5 ml minimal essential medium (MEM) vitamins, and 250 mg glucose, all per 500 ml medium. If contamination was suspected refobacin (2.5 mg, Merck, Darmstadt, F.R.G.) was added (antimycotics were not used).

Characterisation of cultured cells

If an explanted tumour was histologically diagnosed as carcinoma the epithelial origin of the cultured cells was shown in interference microscopy by polygonal shape and in immunocytochemistry by detecting cytokeratin and other markers [16, 17] (Table 1). If the marker was absent in the cells, the culture was excluded. A maximum of approx. 10% of mesenchymal, cytokeratin-negative cells was accepted in the flasks, since they did not influence the results (data not shown) and were found to be a physiological support for proliferation of primary carcinoma cultures [8].

In sarcomatous tumours the spindle-shaped growth pattern and the immunocytochemical demonstration of related markers (Table 1) was taken as a sign of mesenchymal origin. In melanomas with great cytological variation, S-100 protein, HMB45 positivity or melanin granules were used as markers.

To prove malignancy of the incubated cells the cultures underwent DNA measurement [17] by computer assisted image scanning cytometry (Leitz MPV 2 compact or Olympus CUE III). Only cultures with more than 15% atypical hypertetraploid (> 4,5n) nuclei were included. Diploid and multidiploid (4n, 8n) cultures were excluded as not necessarily malignant.

Drugs

Most drugs were used in the commercially available form. Activated (i.e. hydroxylated) cyclophosphamide and iphosphamide were prepared by the Asta-Werke (Bielefeld) and dacarbazine (DTIC) was activated by applying white light for 30 min [18]. The panel of test substances for the individual cases was made in reference to therapy schedules proposed by clinicians.

Table 1. *Marker substances for immunocytochemical characterisation of cultured cells**

Histogenesis	Marker protein
Carcinomas	Cytokeratin
Gastrointestinal	Carcinoembryonic antigen, CA 19-9, CA 17-1a
Ovary	CA 125
Bronchic	Chromogranin, neuron specific enolase
Breast	CA 15-3, oestrogen and progesterone receptor
Teratomas	Alpha-fetoprotein
Melanomas	S-100, HMB-45
Sarcomas	Vimentin,
Muscle derived	Desmin, actin, smooth muscle antigen,
Fibro-histiocytic	Alpha-1-antitrypsin
Neurogenic	S-100

* If present in the primary tumour as shown by immunohistochemistry.

Each drug was applied in three concentrations (C1, C2, C3) with $C1 = 10^{-1} \times C2$ and $C3 = 10 \times C2$. Concentration C2 was deduced from levels achievable in tumour tissue [18]. It was calculated for each drug, i.e. for those with a half-life $T_{1/2} > 2h$ C2 corresponded with serum level 2 h after application, for drugs with $T_{1/2} < 2h$ C2 corresponded with 10% of serum peak concentration. C2 was selected for doxorubicin (DX) = 0.05 μg/ml, 4-OOH-iphosphamide (IF) = 0.50 μg/ml, cisplatin (CDDP) = 0.50 μg/ml, actinomycin D (ACT) = 0.007 μg/ml, mitomycin C (MIT) = 0.05 μg/ml, 5-fluorouracil (5-FU) = 0.05 μg/ml, methotrexate (MTX) = 0.014 μg/ml, etoposide (VP-16) = 0.14 μg/ml, mitoxantrone (DHAD) = 0.02 μg/ml, vincristine (VCR) = 0.005 μg/ml, dacarbazine (DTIC) = 0.35 μg/ml, and 4-OOH-cyclophosphamide (CYC) = 0.07 μg/ml.

Determination of drug effect

Chemoresistance was tested in early passages (P2–P4). For testing 12 × cluster plates (Costar) were used. Into each well 1×10^4 cells/cm^2 were seeded, followed by a 3-day period of settling and propagating. At the beginning of the 3-day test period the number of cells (n) was determined in six wells and defined as baseline n_{prae}. Then the drugs were added at concentrations C1, C2 and C3. At the end of a test the cells were counted in six control wells without drug addition (n_{post}) and in the test plates with cytostatics (n_{C1-3}). All incubations were done in triplicate. For assessment of reproducibility 10 randomly selected assays were repeated five times, resulting in an interassay variance of less than 10%.

Increase of cell number in control wells was calculated by subtraction on $n_{post} - n_{prae}$; increase or decrease in test incubations was expressed as relative growth (G_{rel}) of controls and estimated by the formula

$$G_{rel} = \frac{n_{Ci} - n_{prae}}{n_{post} - n_{prae}} \times 100$$

where, n = number of cells, $i = 1, 2, 3$.

In vitro/in vivo *correlations*

The definition of sensitivity versus resistance was determined empirically. It was based on the first 50 retrospective correlations (Fig. 1, open circles). Best predictive accuracy was found if the proliferation rate of the untreated controls (expressed as n_{post} divided by n_{prae}) was related to G_{rel} of the treated cultures and expressed as response index (RI):

$$RI = \frac{n_{post}}{n_{prae}} \times G_{rel}.$$

Clinical response to chemotherapy was assessed in accordance with the WHO Handbook [19] as complete remission (CR), partial remission (PR), no change (NC) and progressive disease (PD). This was based on direct measurement of tumour volume, computer tomography, sonography, scintigraphy and, if available, tumour marker levels. The period of observation was at least 4 months, with a mean of 9 months.

In vitro/in vivo correlations were evaluated as follows: in single-agent therapy a correlation was established if a tumour was sensitive *in vitro* and showed PR or CR *in vivo* (S/S), or if resistance was deduced from the assay and if NC or PD occurred clinically (R/R). A discrepancy was determined if a tumour was estimated as sensitive *in vitro* and the clinical outcome was NC or PD (S/R), or if a tumour appeared resistant *in vitro* and a response was found clinically (R/S).

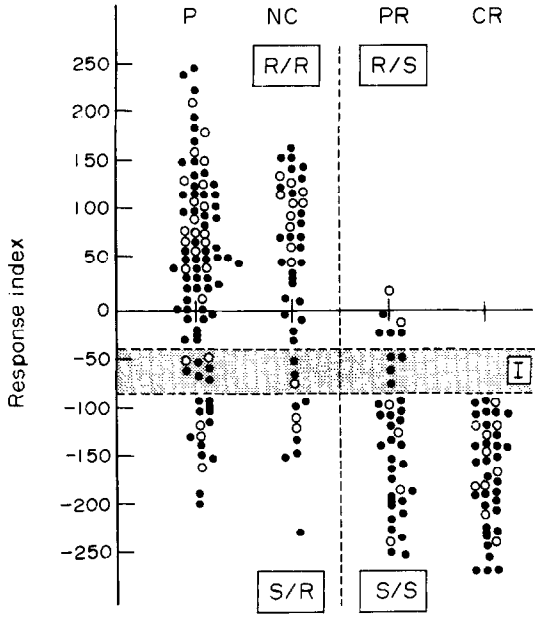

Fig. 1. Clinical response in relation to test results as shown for 214 *in vitro/in vivo* correlations. The first 50 retrospectively determined cases are indicated as open circles. The response index is explained in the text. S = sensitive, R = resistant, I = intermediate, P = progress, NC = no change, PR = partial remission, CR = complete remission.

For drug combinations a correlation was assumed if the MP-assay predicted sensitivity for one of the clinically applied drugs and if the tumour responded with PR or CR (S/S), or if the assay predicted a tumour to be resistant to all cytotoxic agents tested and *in vivo* NC or PD was observed (R/R). Combinations other than these were considered discrepant (S/R or R/S).

Drug response was evaluated at C2 in solid tumours considered for systemic therapy and at C3 in effusions considered for intracavitary drug application. Since several drugs were examined *in vitro* and most patients received a combination chemotherapy, the number of *in vitro/in vivo* correlations exceeded the number of cases (see Table 2). Significance was analysed using the χ^2 test.

RESULTS

Tumour material and cell cultures

Four-hundred and fifty-two specimens of different origin (breast, oesophagus, stomach, pancreas, colon, bronchus, ovary, kidney, testis, soft tissues, melanomas, tumours of childhood) were sent to our laboratory. Of these, 243 were solid tumours (131 primary tumours, 112 metastases) and 209 were ascites or pleural effusions. None of the patients had received prior chemotherapy. In all cases the diagnosis was made by histology or cytology. Three-hundred and twenty-one (71%) proliferating cell cultures that fulfilled the characterisation criteria could be established.

Table 2. Correlations between in vitro *results and clinical outcome**

	Agreement		Discrepancy	
	S/S	R/R	S/R	R/S
	in vitro/in vivo			
200 correlations†	69‡	103‡	22‡	6‡
Predictive accuracy	75.8%‡	94.5%‡	24.2%‡	5.5%‡
	86.0%		14.0%	

S = sensitivity, R = resistance.

* 14 results evaluated as intermediate are not included (see text).

† 104 cases were treated with one or several cytostatic agents, resulting in 200 individual correlations.

‡ $P < 0.001$ evaluated by χ^2 test.

Cytostatic effectiveness in vitro

A typical dose–response curve for the evaluation of drug effects is shown in Fig. 2. The primary tumour was diagnosed as pancreatic carcinoma (KiPP90-12). The applied drugs resulted in dose-dependent growth inhibition with distinct variations. On the basis of the described RI, at concentration C2 (see Materials and Methods) the tumour was determined sensitive to CDDP, DHAD, and IF, intermediate, i.e. not clearly predictable, to MIT and DX and resistant to 5-FU, MTX, and ACT.

Summarising all assay data, it became evident that tumours derived from one organ with analogous histogenesis (e.g. adenocarcinoma of the pancreas) often expressed great variations in drug response.

In vitro/in vivo *correlation*

Out of 321 assayed cases 104 could be used for comparing *in vitro* data with clinical follow-up, resulting in 214 comparisons (Fig. 1). The cohort of the first 50 retrospective correlations are marked by open circles to show the basis on which cut-off lines of the *in vitro* RI was defined. The tumours were determined as resistant when the RI was > -40, as intermediate when the RI was between -41 and -80, and as sensitive when the SI was < -81.

In 172 of 200 correlations the MP assay indicated the clinical response correctly (S/S and R/R) resulting in an overall predictive accuracy of 86% (Fig. 1 and Table 2). Twenty-eight correlations (14%) were found to be discrepant, of these 22 *in vitro* results predicted sensitivity which was not found *in vivo* (false positive, S/R) and six correlations appeared to be false negative (R/S). Thus, the MP-assay gave a prospective evaluation of tumour

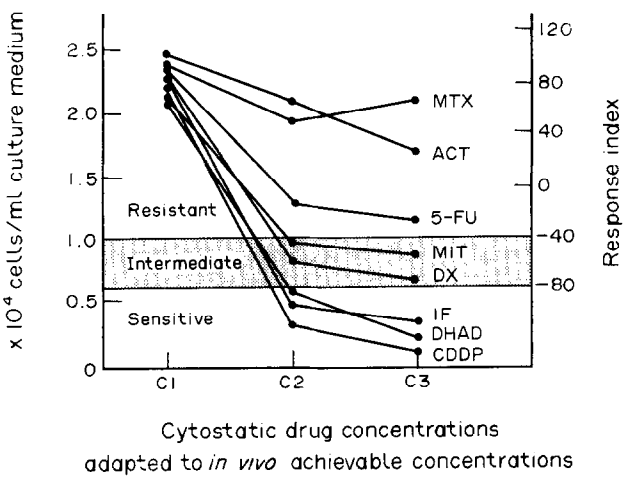

Fig. 2. Representative growth inhibition curve of the monolayer proliferation assay to predict cytotoxic drug-related growth modulation of malignant tumours *in vivo*. The concentration C2 is adapted to clinically achievable levels (C1 = 0.1 × C2, C3 = 10 × C2; see Materials and Methods). The response index (right *y*-axis) is explained in the text. The evaluation of response is made at C2. All points represent mean of triple cultures, S.D. < 10%.

reaction with true positive results of predicting resistance in 94.5% and of sensitivity in 75.8%. The data agree significantly with clinical outcome ($P < 0.001$, χ^2 test). Fourteen test results lay in the intermediate range without allowing prediction of response. Clinically, they showed 8 PR, 9 NC, and 4 PD.

217 cases could not be evaluated because of exit prior to therapy ($n = 60$), no chemotherapy was performed ($n = 72$), left clinical control ($n = 37$), did not complete the 4 month period of observation ($n = 48$).

DISCUSSION

Since no clear correlation exists between organ origin, histological type, grading, and other tumour characteristics on the one hand and drug response on the other, selection of the appropriate xenobiotic for the individual lesion is often difficult [1, 14, 20]. Hence, it is necessary to refer to clinical studies. These, however, include many factors that cannot be standardised and thus have only limited relevance for the individual patient. A reliable test for predicting chemoresistance/chemosensitivity of a tumour would be of great importance [18]. This is especially true for rare tumours, tumour recurrences, and metastases with unknown vulnerability.

In order to verify relevance of the MP-assay described here, each *in vitro* result was compared with the clinical outcome. Based on the criteria described for mono- and multiagent therapy, the *in vitro/in vivo* correlations revealed an overall agreement of 86%. Similar to other test systems [4, 6–8], prediction of resistance is associated with a greater accuracy (94.5%) than prediction of sensitivity (75.8%). The discrepancies may be explained by cellular heterogeneity within tumours and their metastases as well as by selective *in vitro* growth, differing drug metabolism or non-simulatable *in vivo* conditions such as vascularisation, tissue hypoxia, etc. These are intrinsic limitations of *in vitro* assays, which in our opinion will prove difficult to overcome in the near future. Nonetheless, the results reported by us and others [3, 9–12] verify assay-based considerations when therapeutic strategies are planned [21–23]. This is further supported by clinical trials showing [24–26] prolonged survival time if chemotherapy was conducted following predictive test systems. In the selection of second-line or metastasis chemotherapy *in vitro* assays is under discussion [27, 28].

In the first 50 cases examined, a tumour was determined to be resistant or sensitive based on the retrospective comparison of *in vitro* results with clinical follow-up. Remarkably, only distinct *in vitro* cytotoxicity, i.e. reduction of the number of cells below that at the beginning of the test, was correlated with a clinical response of partial or complete remission. In other test systems [5–7, 26, 29] often only growth inhibition and not the partial death of cultures, colonies or experimental tumours is used as the criterion for discriminating between resistance and sensitivity. This may be less efficient for obtaining relevant results.

Furthermore, it appeared to be important that not only growth reduction but also culture doubling times had to be considered when *in vitro/in vivo* correlations were established. This was taken into account by introducing a response index which reflected the following: to reach similar test results slowly growing cultures had to be inhibited more intensively than fast growing ones. This observation could be explained by the propensity of most cytostatics to attack tumour cells during mitoses. Fast growing tumours with relatively short cell cycles are more vulnerable to cytostatics than slowly growing ones. The clinically established higher drug sensitivity of rapidly proliferating tumours supports this thesis.

Different pharmacological properties of anti-cancer agents are a major problem of *in vitro* assays. Mode of application, metabolism, elimination and interaction with plasma proteins can be simulated only partially. In addition, drug distribution differs from tissue to tissue and from tumour to tumour. In the described assay drug effectiveness was determined at concentrations which are approximately attainable in the microenvironment of tumour cells *in vivo* and thus have therapeutic relevance [18]. The 3-day period for test incubations was chosen, since a majority of antineoplastic agents are applied sequentially over several days and thus are effective at least for this period.

Alternative test systems for predicting resistance without cell culture procedures are under discussion. These systems focus on the identification of specific resistance related mechanisms (for review see ref. 30), such as marked expression of P-glycoprotein, glutathione-*S*-transferase, topoisomerases I and II, etc. However, the described mechanisms may have limited predictive utility, since they are in varying degrees responsible for the chemoresistance of a given tumour and the net effect of all mechanisms can hardly be determined. Thus, it seems to us more advantageous to determine the net result of all factors involved using *in vitro* assays.

The satisfactory *in vitro/in vivo* correlation of cytostatic drug activity on solid human tumours demonstrated in our MP assay opens the way for its application in preclinical screening assay for new compounds with suggested anticancer potency. Such screening assays based on primary cell cultures may represent the actual drug–cell interaction more closely and thus may have greater predictive value than systems based on established cell lines.

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Antitumour Activity of an Immunoconjugate Composed of Anti-human Astrocytoma Monoclonal Antibody and Neocarzinostatin

Seiji Kondo, Shouji Nakatsu, Harumi Sakahara, Hisataka Kobayashi, Junji Konishi and Yuziro Namba

Neocarzinostatin (NCS) linked to the thiol group on the hinge region of the Fab' fragment of GA-17, a murine monoclonal antibody reacting with tyrosine-specific phosphorylated antigens, which are exclusively expressed on the cell surface of human astrocytomas, was evaluated for *in vivo* activity. GA-17-NCS immunoconjugates significantly suppressed the growth of human malignant glioma cell line U87-MG subcutaneous xenografts in nude mice until day 50 when administered intravenously into the tail vein. Disulphide- and thioether- linked GA-17-NCS were nearly equipotent immunoconjugates, but thioether-linked GA-17-NCS was more effective than disulphide-linked conjugates with 250 U/kg NCS content on day 50 ($P < 0.05$). Thioether-linked GA-17-NCS was significantly more effective on day 50 than free NCS with 500 U/kg or 250 U/kg NCS content ($P < 0.05$, $P < 0.01$, respectively). These results suggest that GA-17-NCS may prove useful in the treatment of human malignant gliomas.
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INTRODUCTION

MALIGNANT GLIOMAS, the most common primary malignant tumours of the brain, are rapidly fatal. The best treatment currently available (surgery, radiation therapy and systemic chemotherapy) results in median survival times of less than 1 year [1].

In recent years, monoclonal antibodies (Mab) recognising tumour-associated cell surface antigens have been widely utilised as tumour-specific carriers for cytotoxic agents such as toxins, radioisotopes, and anticancer drugs [2–5]. The efficacy of immunoconjugates depends upon both the Mab and drug components, and an immunoconjugate may potentially have advantages over