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Inter-experiment Variation and Dependence on Culture Conditions in Assaying the Chemosensitivity of Human Small Cell Lung Cancer Cell Lines*

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Abstract—*Sensitivity of five human small cell lung cancer cell lines to doxorubicin was assessed by a double layer agar technique using two different bottom-layers. Neither of the bottom-layers provided proportionality between numbers of cells plated and numbers of colonies, but they were correlated by a logarithmic function.*

Even after correction for lack of proportionality the two assay systems provided significantly different dose–response curves.

The stability of the chemosensitivity was tested after 25–30 *weeks continuous* in vitro *culture or prolonged storage in liquid nitrogen. One cell line underwent significant changes after continuous* in vitro *culture whereas the cell lines tested after prolonged storage in liquid nitrogen showed only minor changes.*

It is concluded that instead of considering the concentration necessary to achieve a certain degree of cell kill (e.g. ID_{50}*) in one experiment on one cell line, dose–response curves obtained on several cell lines in different assay systems should be used in the evaluation of new drugs.*

INTRODUCTION

CLONING of mammalian cells were introduced in 1955 by Puch and Marcus [1] using irradiated cells to supply conditioning factors. Renewed interest was evoked in the culturing of human tumor cells, when Salmon *et al.* in 1978 [2] introduced the clonogenic assay as a potential clinically applicable sensitivity test. While the validity of the clonogenic assay for use in predicting clinical response to drug therapy remains to be established [3], the ability of the assay to identify promising agents which would be missed by the conventional *in vivo* screening systems indicates that the assay may be a useful addition to the NCI drug-screening program [4].

This investigation was initiated to assess the potential of "*in vitro* phase II testing" of drugs in a panel of cell lines established from patients with small cell carcinoma of the lung (SCCL). The inter-experiment variation was assessed, and the sensitivity patterns obtained were compared using two different bottom-layers. Finally, the stability of the sensitivity after continuous *in vitro* culture or prolonged storage in liquid nitrogen was investigated.

MATERIALS AND METHODS

Cell lines

All cell lines were established from patients with SCCL. NCI-H69 and NCI-N592 were provided by Desmond N. Carney, NCI-Navy Medical Oncology Branch, Bethesda, U.S.A.

OC-TOL, OC-ROL, OC-NYH were received from Loe de Ley, Laboratory of Clinical Immunology, Gronningen, Holland.

All cell lines express the properties associated with cells of the APUD series, including neurosecretory granules, high levels of L-dopa decarboxylase and polypeptide hormone secretion [5].

Growth behaviour and DNA content are shown in Table 1. All cell lines were free of mycoplasma contamination. The genetic stability of the cell lines was checked by flow cytometric DNA analysis every month.

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Single cell suspensions were achieved by passing the aggregates through 18G needles. Viability was assessed by dye exclusion.

Tissue culture media

All cell lines were maintained in liquid culture in RPMI-1640 supplemented with 10% fetal calf serum (FCS) L-glutamin (0.29 g/l), streptomycin (50 mg/l) and penicillin (200,000 IU/l). Medium and serum were obtained from GIBCO Ltd., Paisley, Scotland.

Bottom-layers

Experiments were performed in agar culture systems on two different bottom-layers: 1. "SRBC" bottom-layers were prepared as a modification of the bottom-layers described by Engelholm *et al.* [6]. One millilitre of Eagles minimal essential medium (MEM) containing MEM amino acids, MEM vitamins, L-glutamine (0.16 g/l), glucose 10% (5 ml 1-1), streptomycin (28 mg/l), penicillin (100,000 IU/l), 0.5% agar (Difco), 2.5% (by vol) sheep red blood cells (SRBC) and 5×10^{-5} M mercapto-ethanol was poured in 35 mm plastic Petri dishes and allowed to solidify. The SRBC bottom-layers were stored for up to 1 week at 4° C. 2. "RPMI" bottom-layers were prepared afresh every day by adding 10% of a 3.3% agar in PBS to the above-mentioned RPMI-1640 with FCS. 1.5 ml of this mixture was poured into 35 mm Petri dishes and allowed to solidify before they were stored in the incubator to equilibrate the pH. the pH.

Drug

Doxorubicin (Farmitalia, Carlo Erba s.p.a.) was dissolved in distilled water and 100 × final concentrations dilutions were made with tissue culture media.

Clonogenic assay

The cells were suspended in tissue culture media with 0.3% agar and plated with 1 ml on top of each bottom-layer. After solidifying, 1 ml of tissue culture media was added to prevent drying. After incubation for 3 weeks at 37° C in a 7.5% CO_2 humidified atmosphere of air, the dishes were examined for tumor colonies using a dissecting microscope. Colonies were defined as a collection of cells with a diameter of at least 4 times that of a single cell ensuring a content of at least 64 cells [7]. On plates with more than 300 colonies only 3 preselected areas of 1/10 of the plate were counted.

To test the relationship between the number of plated cells and the resultant number of colonies, increasing numbers of cells were set up in the clonogenic assay. These experiments were performed at regular intervals throughout the experimental period (18 months) to determine the variation of the constants used for correction.

Drug exposure

From the dilution curves the number of cells that would give a yield of approx. 2000 colonies was chosen and a suspension of this number of cells per ml was prepared. For drug exposure 3.5 ml of this mixture was aliquoted into test tubes, 35 µl of the drug solutions, or tissue culture media in the control tube, were added. The tubes were left horizontal in the incubator for 1 hr, the cells were hereafter spun down at 170 ***g*** for 5 min and washed twice with PBS. Of the agar tissue culture media, 3.5 ml was added to the cell pellet and three bottom-layers were seeded with one ml in each yielding triplicate determination of each concentration. Because of the cell loss by these manipulations, determined in previous experiments to be approximately 50%, these procedures were estimated to give a yield of approx. 1000 colonies on each of the control plates.

Data analysis

Linear regression analysis was used on the logarithmic transformed data as well as the raw data to estimate the parameters describing the relationship between the number of colonies (dependent

Table 1. *Source,* in vitro *growth behaviour and DNA content of the used cell lines*

Cell line	NCI-69	NCI N-592	OC ROL	OC TOL	OC NYH
Established from	Pleural effusion	Bone marrow	Pleural effusion	Pleural effusion	Pleural effusion
In relation to behaviour	After	After	After	Before	Before
Growth chemotherapy	Suspension	Suspension	Suspension	Suspension	Monolayer + suspension
DNA content	approx. 0.9	1.52	1.75	1.39	1.39

The DNA content is indicated relative to the content of normal human diploid cells in G_1-phase. The content of NCI H-69 is only approximate as the DNA content is almost equal to one of the internal references.

variable) and the initial number of cells (independent variable). Analysis of variance was used to test hypotheses on the variation between the two bottom-layers and between the different cell lines.

RESULTS

Dilution experiments

When the fraction of surviving colonies is used for assaying the number of surviving cells, proportionality between the number of cells plated and the number of colonies formed is often assumed. Figure 1 depicts representative experiments showing the number of colonies as a function of the number of cells plated on the two different bottom-layers. OC-Rol is only shown on SRBC as it did not form colonies when grown on the RPMI bottom-layer.

Data from both culture systems were fitted so that the number of colonies was a linear function of the number of cells plated. The fits were weighted least-squares as homoscedacity could not be assumed and r^2 (r = coefficient of determination) was greater than 0.95 in most cases. However, the hypothesis of proportionality was rejected in almost all cases ($P < 0.05$). The major reason for this was clearly the relatively poor fits to the data in the region where 1000 cells or less were plated. Indeed, the hypothesis holds in many cases in the range of more than 10^3 to 10^4 cells seeded. The plating efficiency apparently declined when more than 10^4 cells were seeded.

In order to improve the fits for the lower number of cells seeded, the data was log transformed and then the best linear relationship between the number of colonies and the number of cells plated was estimated. Now the value of r^2 was greater than 0.97 in all cases.

The results are shown in Fig. 1 which also includes the best fit assuming proportionality. The predicted number of colonies is given by the following equation:

$$N = AX^B \qquad (1)$$

where N is the predicted number of colonies, A and B are constants estimated for each bottom-layer in each experiment and X is the number of cells plated. Because the values of B are used to correct the survival curves for lack of proportionality (see below) the variation of B was investigated. The most pronounced variation was found between the two bottom-layers ($P < 0.001$) where B was significantly below one on SRBC (mean 0.89 S.D. 0.16 in 18 experiments) and above on RPMI (mean 1.24 S.D. 0.20 in 14 experiments). B values below one implies that the plating efficiency (PE) increased with decreasing cell numbers plated whereas B value above one implies decreasing PE with decreasing cell numbers. Although B varied between the individual experiments and technicians, any systematic variation due to time was not found. In addition, the variation in B between the cell lines was not significantly larger than the variation within each cell line on each bottom-layer. It was concluded that the variation of B necessitates correction of survival curves with simultaneously-performed dilution curves.

Chemosensitivity

Before considering whether the sensitivity pattern is dependent on the culture conditions it is important to assess the inter-experiment variation. Dose–response curves for doxorubicin were determined twice in successive passages for each cell line. The surviving fractions were calculated by dividing the number of colonies on the treated plates with the number of colonies on the control plates (Fig. 2). It is seen that the inter-experiment variation is negligible compared to the effect of different bottom-layers.

Instead of calculating the surviving fraction as described above the corresponding number of viable cells plated on each concentration can be read from simultaneously performed dilution curves and divided by the number of cells on the control plates (also read from the dilution curves). This correction for lack of proportionality can be done mathematically:

$$S_t = \frac{N_t}{N_o} = \frac{AX_t^B}{AX_o^B} = \left(\frac{X_t}{X_o}\right)^B = Z_t^B => Z_t = (S_t)^{1/B} \qquad (2)$$

where N, A, X, B are defined in eqn. 1 and

S_t = surviving fraction based on number of colonies at drug concentration t

Z_t = surviving fraction based on number of cells at drug concentration t.

In Fig. 3 the corrected dose–response curves are shown. It is seen that when the data are corrected for this lack of linearity the differences in dose–response curves obtained on the two bottom-layers declines although there still is a significant difference between the sensitivity patterns obtained in the two growth conditions.

Stability of chemosensitivity

In Fig. 4 the dose–response curves obtained initially are compared with the results obtained after 40–50 weeks of storage in liquid nitrogen or 25–30 weeks continuous culture. It appears that the sensitivity of the cell lines is fairly stable, although there is a trend towards decreasing sensitivity in the late experiments. One cell line (OC-ROL) appears more sensitive in the late exper-

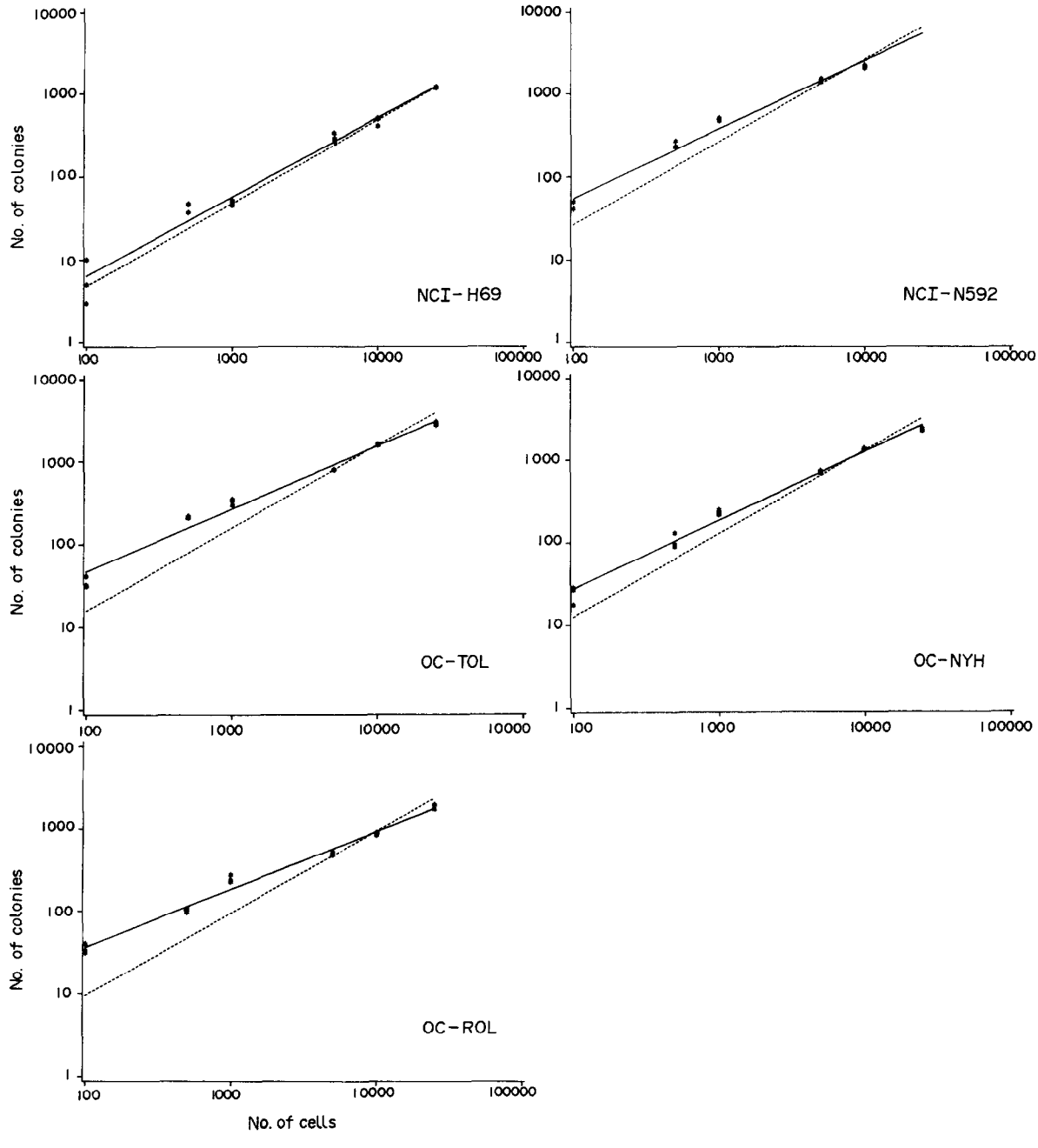

Fig. 1 (*a*). *Number of colonies as a function of the number of cells plated on the two different bottom-layers (Fig.* 1*a*: *SRBC, Fig.* 1*b*: *RPMI). Each point represents the results of one plate. The solid line shows the best fit to the log transformed data. The dotted line shows the best fit (weighted) assuming proportionality. This line will in all cases have a slope equal to one in this double logarithmic plot. In all cases, the dotted line can be seen to fit the data in the middle range of the number of cells seeded, indicating proportionality here.*

iment than in the one performed initially. This cell line is also showing the greatest inter-experiment variation, both within a few sub-cultures (Fig. 2) and after 28 sub-cultures.

DISCUSSION

The use of cell lines offers the opportunity to assess the inter-experiment variation of the assay since there is no shortage of cells as encountered when working with fresh tumor samples.

In this study the inter-experimental variation was within the experimental error when the experiments were repeated after a few sub-cultures (Fig. 2). Two of three cell lines showed no significant changes in sensitivity pattern after 25–30 weeks continuous *in vitro* culture, whereas one cell line (OC-ROL) underwent a significant change (Fig. 4). This cell line also showed the greatest inter-experiment variation. This might indicate that OC-ROL contained two or more sub-populations

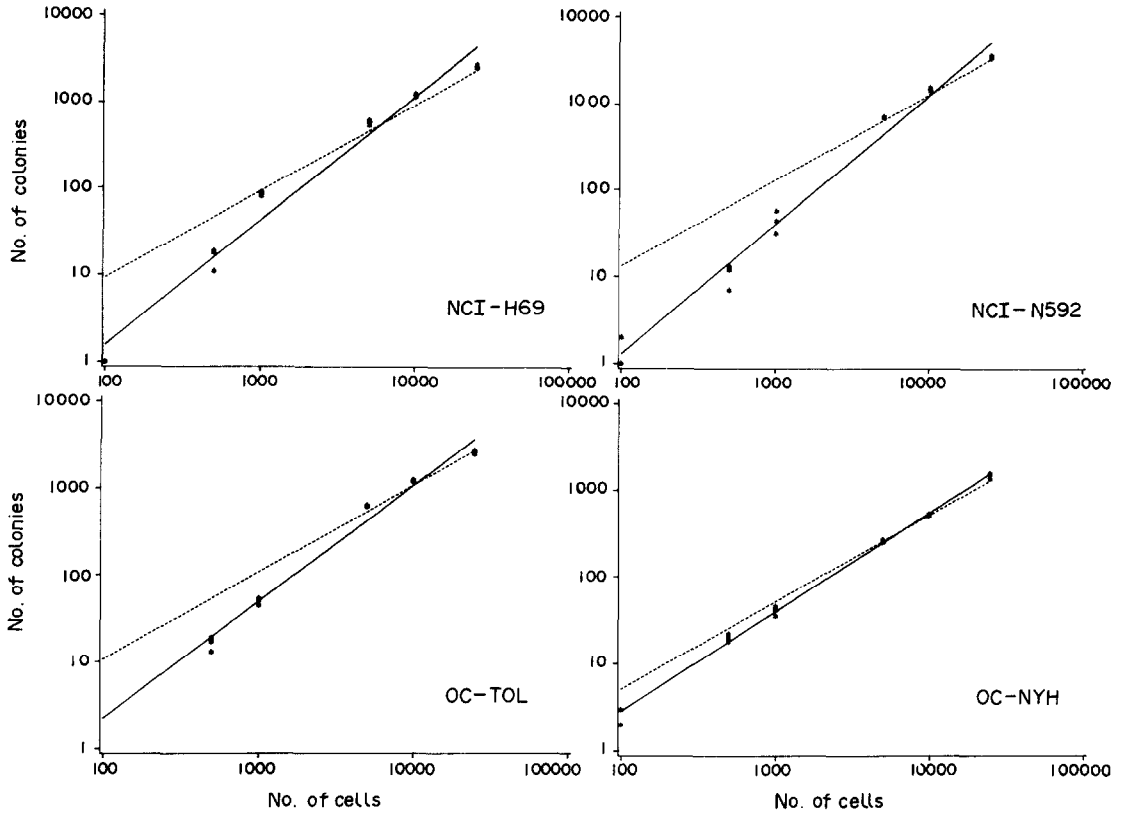

Fig. 1(b). Number of colonies as a function of the number of cells plated on the two different bottom-layers (Fig. 1a: SRBC, Fig. 1b: RPMI). Each point represents the results of one plate. The solid line shows the best fit to the log transformed data. The dotted line shows the best fit (weighted) assuming proportionality. This line will in all cases have a slope equal to one in this double logarithmic plot. In all cases, the dotted line can be seen to fit the data in the middle range of the number of cells seeded, indicating proportionality here.

with different sensitivity. If so these sub-populations must have identical cellular DNA-content since the DNA-index was stable. (Data not shown.)

When the cell lines were retested after prolonged storage in liquid nitrogen they showed only minor changes in sensitivity pattern. This observation is indicative of the above-mentioned variation being real and not due to instability of the assay. Ideally all of the cell lines should have been retested after prolonged storage in liquid nitrogen, but both OC-ROL and OC-TOL failed to grow after thawing. Stability of the sensitivity pattern of a heterogenous cell line in the frozen state would require no population selection. Most investigators consider that frozen storage does not influence population selection, but this may vary according to the parameters used for evaluation [8].

The present results show that chemosensitivity data obtained on continuous cell lines may be misleading. Valid conclusions concerning the sensitivity of a certain tumor type to a specific drug cannot be drawn on the test of a single or a few cell lines in only one sub-culture number. For each tumor type a panel of different lines should be examined [9], and the stability of the sensitivity pattern should be tested at regular intervals.

One potential source of artifact in the clonogenic assay is a non-linear relationship between the number of colonies obtained and the number of cells plated [10]. Our results demonstrate that the linearity requirement might be claimed to be fulfilled with high correlation coefficients although the results obtained relate poorly with the fitted curves when less than 1000 cells are plated. A lack of proportionality in this range will result in artifactual dose–response curves, when drugs yielding a high cell-kill are tested. The description of the dilution experiments by Equations (1) and with B-values different from one provides well-fitting curves in the entire range of cells plated.

The use of dilution experiments for a mathematical correction for lack of proportionality is done on the assumption that the PE of a small number of cells growing alone is identical to the PE of the same number of cells surrounded by a large number of non-dividing cells. Although it still is unclear whether this assumption is fulfilled, the potential of such a correction is indicated by the fact that the difference between the surviving curves, obtained in the two culture systems, is minimized after correction.

If the assay is used to compare drugs in simultaneously-performed experiments, the lack of proportionality will affect the dose–response curves

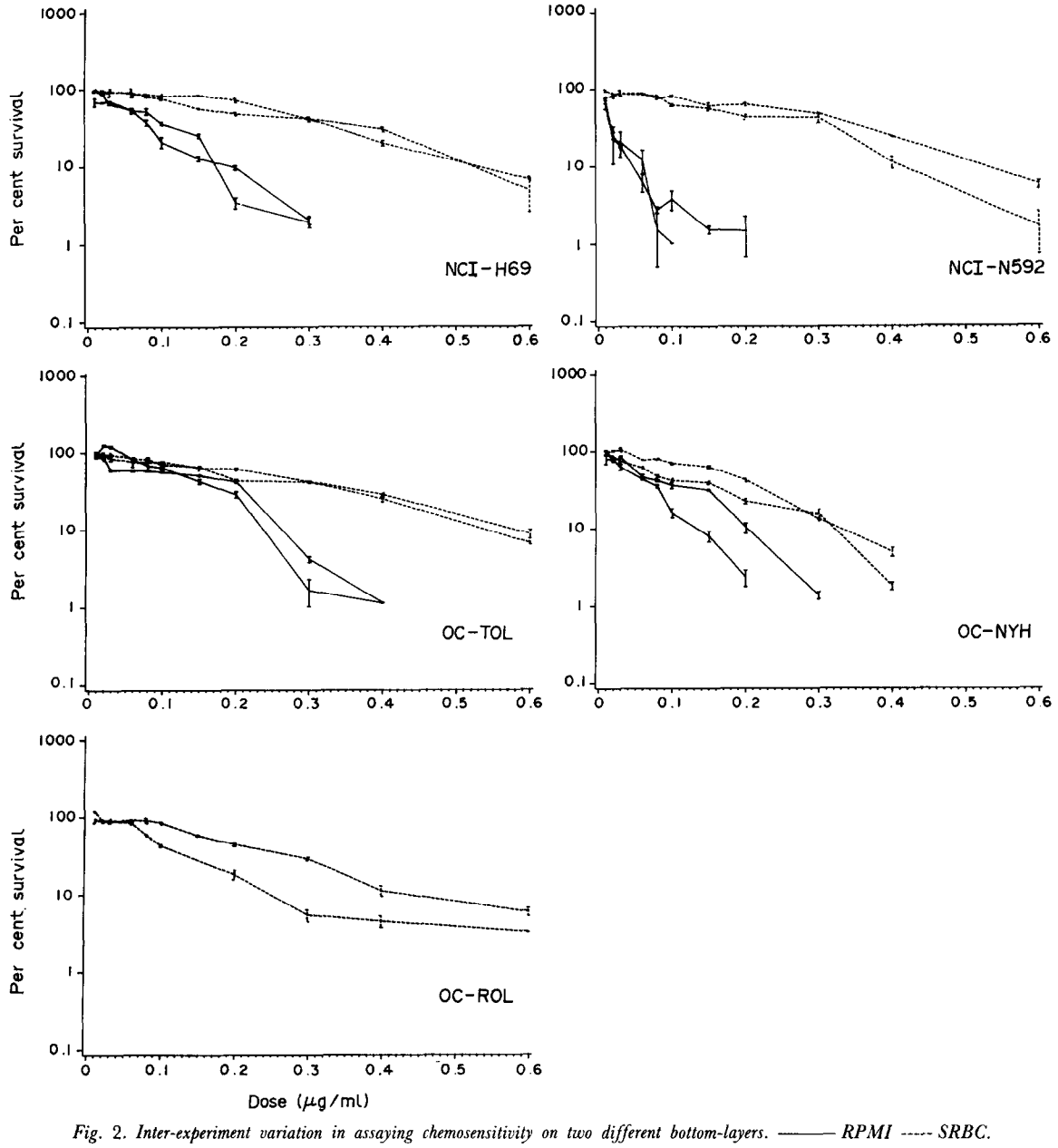

Fig. 2. Inter-experiment variation in assaying chemosensitivity on two different bottom-layers. ——— RPMI ----- SRBC. Each experiment was done in triplicate and was repeated. Plates without colonies were omitted. The surviving fractions were calculated by dividing the number of colonies on the treated plates with the number of colonies on the control plates. Bars represent 2 S.E.M.

identically and correction is unnecessary. Whereas the influence of lack of proportionality in experiments performed at distinct time or under varying culture conditions can not be assumed to be identical, and comparison of uncorrected data might thus be misleading.

Growth of tumor cells can be induced by different agents. When Puch and Marcus [1] first cloned mammalian cell lines *in vitro* they used irradiated feeder cells. Courtenay [11] added rat blood cells (RBC) as growth inducers. When Hamburger and Salmon [12] introduced their clonogenic assay, growth was promoted by either human type O erythrocytes or medium conditioned by the adherent spleen cells of mineral oil-primed BALB/c mice. Since these initial reports on the cloning of tumor cells many investigators have tested the sensitivity of numerous tumors. However, neither the definition of, nor the method for, determining *in vitro* sensitivity are standardized and vary among the laboratories [13]. One widely-used practice is to incubate the cells for 1 hr with 1/10 the peak plasma concentration obtained in patients. The *in vitro* sensitivity is then defined as a 70% or greater reduction in survival of tumor colony-forming units [14]. The use of a single drug concentration is problematic, since the achievable levels of anticancer drugs vary from patient to

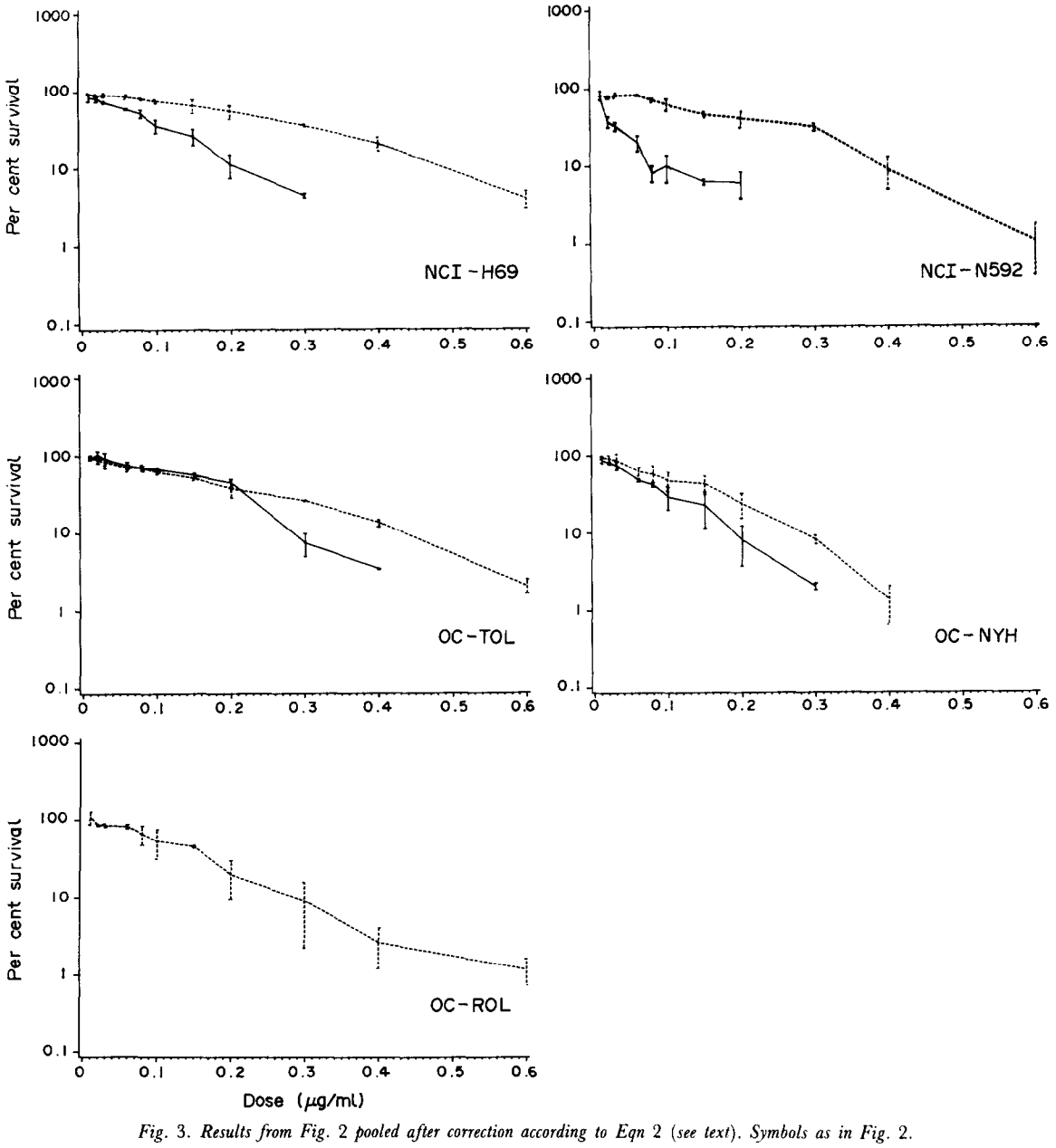

Fig. 3. Results from Fig. 2 pooled after correction according to Eqn 2 (see text). Symbols as in Fig. 2.

patient and since the drug concentration entering the tumor cell in single cell suspensions may differ from that *in vivo* [14]. Another problem is the arbitrary choice of the 1 hr incubation especially when cell-cycle-specific drugs are tested.

The present investigation has shown that the sensitivity pattern is dependent on the culture condition. This problem is only sparsely investigated, although many tumors could be sensitive in one system and resistent in another according to the above-mentioned definition with 70% cell-kill at one-tenth of the peak plasma concentration. Tveit *et al.* [15] have compared the chemosensitivity of human melanomas in a system similar to that of Hamburger and Salmon [16] with a system essentially like that of Courtenay and Mills [17]. They found that the tumor cells appeared to be more sensitive to 3 of 4 drugs in the system without RBC than in the system with RBC. Tveit *et al.* found no linearity between the number of cells plated and the colonies formed in the system of Hamburger and Salmon.

This lack of linearity opens the possibility of the number of surviving clonogenic cells being reduced to the level at which there is no longer proportionality between cell numbers and colonies formed after exposure of the cells to the cytotoxic drugs. The present investigation showed lack of linearity when SRBC was added, contrasting Tveit *et al.*'s data. When the data are corrected for this lack of proportionality there still is a significant difference between the sensitivity patterns obtained with the two culture conditions. This difference might be explained by the superiority of the culture

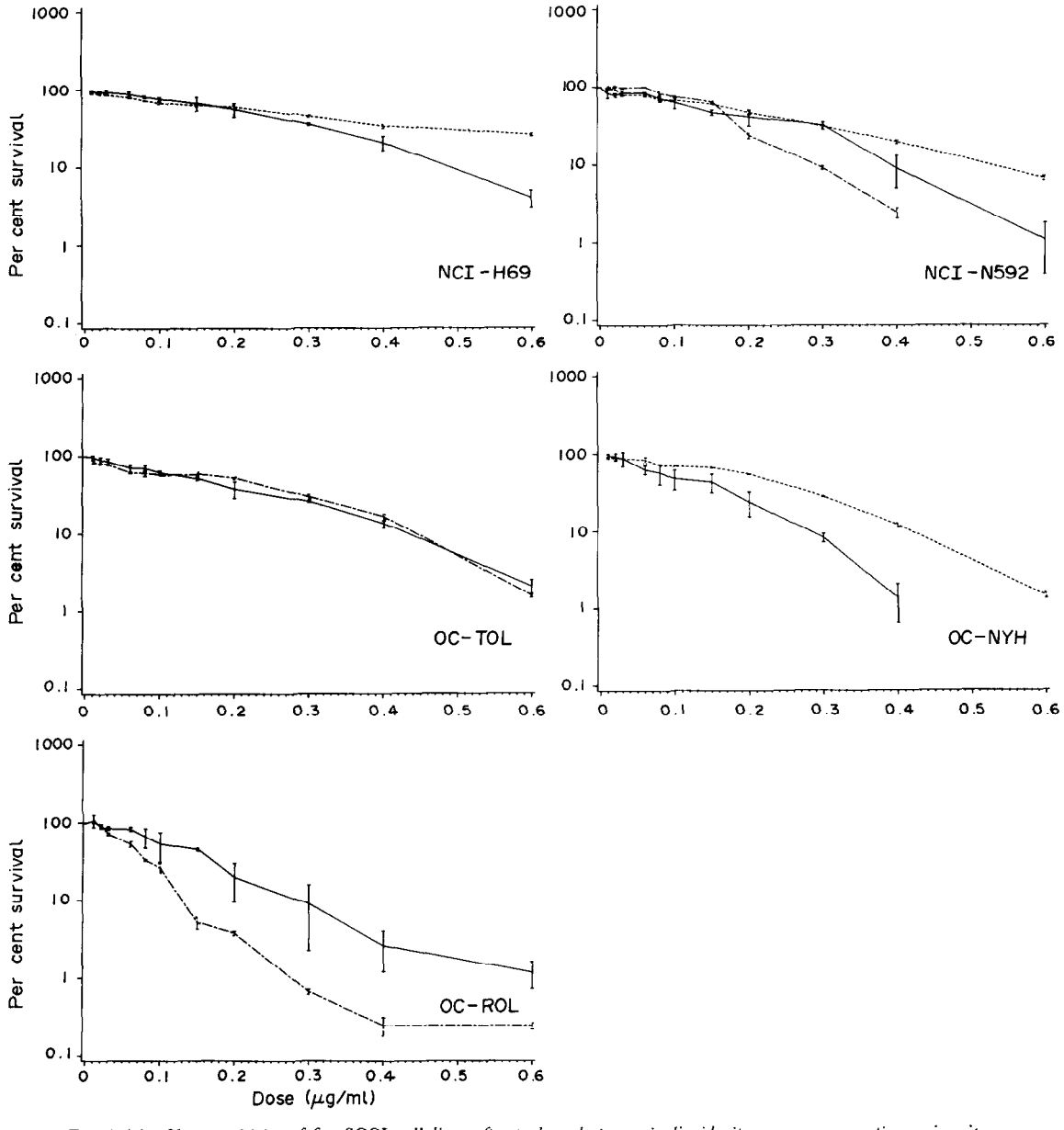

Fig. 4 (*a*). *Chemosensitivity of five SCCL cell lines after prolonged storage in liquid nitrogen* ----- *or continuous* in vitro *culture* –.–. *For comparison the results from Fig.* 3 *are included* ——. *The cell lines were tested on SRBC bottom-layers* (*Fig.* 4*a*) *and RPMI bottom-layers* (*Fig.* 4*b*).

NCI — *N* 592 *was tested after* 47 *weeks in* N_2 *and after* 30 *weeks* in vitro.
NCI — *H* 69 *was tested after* 40 *weeks in* N_2
OC — *NYH was tested after* 49 *weeks in* N_2
OC — *ROL was tested after* 25 *weeks* in vitro
OC — *TOL was tested after* 30 *weeks* in vitro

condition in the system with SRBC, where partially damaged cells recover, whereas they die in the system without SRBC.

Gupta and Krishan [18] have compared the sensitivity of a human melanoma xenograft incubated at 5 and 20% O_2. Incubation in 5% O_2 increased the plating efficiency and enhanced the cytotoxicity for all the drugs tested. This enhanced cytotoxicity is in conflict with the results of Tveit *et al.* Gupta *et al.* [19] propose that these differences may be a function of RBC or liquid feeding necessary in the procedure of Courtenay and Mills. Although Gupta *et al.* have not published dilution curves, the discrepancy with Tveit *et al.* could be explained by lack of proportionality. This is suggested by the finding of a significant increase in plating efficiency of tumor cells exposed to low drug concentrations consistently seen in plates incubated in an atmosphere of 20% O_2 [20].

Hill and Whelan [21] have compared the dose–response of one cell line (COLO 205) to doxorubicin obtained in different assays. They

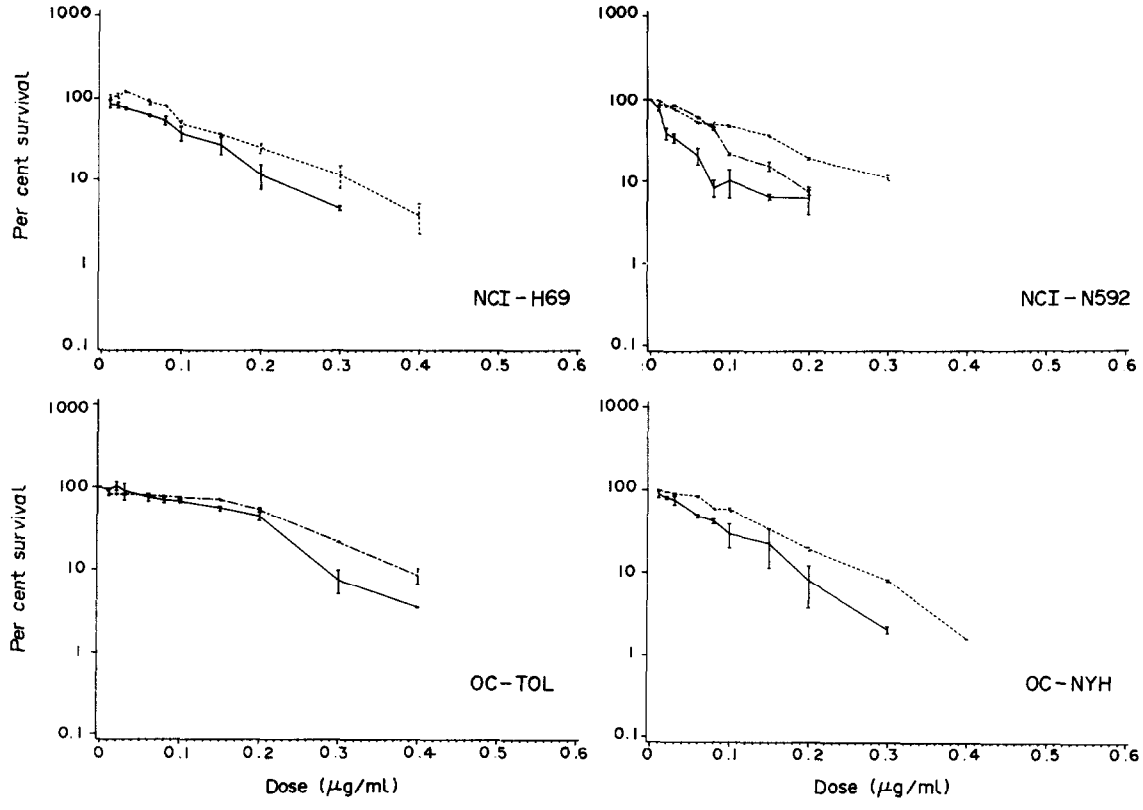

Fig. 4(*b*). Chemosensitivity of five SCCL cell lines after prolonged storage in liquid nitrogen ----- or continuous in vitro *culture* –.–. *For comparison the results from Fig.* 3 *are included* ——. *The cell lines were tested on SRBC bottom-layers* (*Fig.* 4*a*) *and RPMI bottom-layers* (*Fig.* 4*b*).

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found that in this cell line, irrespective of the assay method used, the resulting survival curves were essentially similar.

In vitro testing has been proposed as a method for screening potential anticancer drugs and for assessing the activity of drugs against a given type of tumor. If such methods could be validated they would diminish the need for phase II testing of drugs in patients [10] and thus accelerate the introduction of new drugs.

The results of the present investigation and other similar studies [15, 18, 22] indicate that *in vitro* methods must be tested for linearity and calibrated by correlation with concurrent chemotherapeutic response *in vivo*.

Due to the instability of the sensitivity pattern obtained by some established cell lines and its dependence on the culture conditions, the concentration necessary to achieve a certain degree of cell-kill (e.g. ID_{50}) in one experiment on one cell line is without much predictive value, as well as the comparison of this concentration for different drugs obtained by different experiments. In contrast, the sensitivity patterns obtained by several cell lines established from the same tumor type using a wide range of drug concentrations probably will provide an idea of the efficacy of a new drug against the tested tumor type. One main application of cell lines in sensitivity testing is the comparison of the antitumor effect of different analogues because the parent drug can be used as an internal standard.

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