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Chemosensitivity Testing with Highly Purified Fresh Human Tumour Cells with the MTT Colorimetric Assay

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A major problem associated with the succinate dehydrogenase inhibition (SDI) test using tetrazolium dye (MTT) as a cancer chemosensitivity testing is the contamination of non-malignant cells in the tumour tissues. Highly purified fresh human tumour cells from 44 solid tumours and 24 malignant ascites were used for the MTT assay. The purity of tumour cells was greater than 90% after separation on Ficoll–Hypaque and Percoll discontinuous gradients. The OD_{570} obtained from tumour cells alone was higher than that from non-malignant cells. The chemosensitivity of tumour cells was distinct from that of non-malignant cells. Moreover, the chemosensitivity of highly purified tumour cells was also distinct from that of non-purified cells just separated from tumour tissues. 31 of the 68 patients had evaluable lesions, and received cancer chemotherapy according to the results of MTT assay using highly purified tumour cells. A clinical response was obtained in 10 of the 31 patients (response rate = 32.3%, 5 complete responses, 5 partial responses).
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INTRODUCTION

A RAPID COLORIMETRIC assay was described by Mosmann [1]. This assay determined the ability of viable cells to convert a soluble tetrazolium salt, 3-(4,5-di-methylthiazol-2-yl)-2,5-diphenyl tetrazolium bromide (MTT), into an insoluble formazan precipitate. There have been numerous reports on the MTT assay where tumour cell lines [2, 3], as well as fresh human tumour cells were used as target cells, but without purification of these cells prior to testing [4, 5]. The MTT assay used for chemosensitivity testing of tumour samples, however, should be performed using highly purified fresh tumour cells, since contamination by non-malignant cells affects results and therefore the sensitivity of the test.

This study presents a technique for purification of tumour

cells using discontinuous Ficoll–Hypaque and Percoll gradients. We also present the results of chemosensitivity testing in various fresh human tumour cells, and their correlation with clinical response.

PATIENTS AND METHODS

78 patients with malignant tumours were entered into this study. MTT assays of cells from 68 of the 78 patients were successful for the purposes of the study (success rate: 87.2%), and the reasons for the 10 unsuccessful assays were: few viable cells due to tumour necrosis (3 liver metastases from colon and 1 liver metastasis from stomach), contamination during culture (3 colon cancer), and low OD, less than 0.1, after culture (2 gastric cancer and 1 pancreatic cancer). In 68 patients, surgical specimens were obtained from 44 patients with malignant tumours, including 19 with gastric cancer, 7 with malignant lymphoma, 5 with ovarian cancer, 5 with oesophageal cancer, 4 with pancreatic cancer, 2 with colon cancer and 2 with other cancers. Peritoneal effusions were also collected for analysis from 24 patients with disseminated malignant carcinoma (10 gastric cancer, 5 colon cancer, 3 ovarian cancer, 2 pancreatic cancer and 4 other cancer).

Antitumour drugs

The antitumour drugs tested were mitomycin, 5-fluorouracil (5-FU), doxorubicin, cisplatin and etoposide. Each drug was diluted in complete medium at 10 times peak plasma concentration. These values were mitomycin 10 μg/ml, 5-FU 100 μg/ml, doxorubicin 4 μg/ml, cisplatin 20 μg/ml and etoposide 100 μg/ml, and serial 10-fold dilutions were then performed. The complete medium used consisted of RPMI-1640 (Nissui, Tokyo) supplemented with 10% heat-inactivated fetal calf serum (Gibco), 2 mmol/l L-glutamine, and antibiotics (100 U penicillin/ml and 100 μg streptomycin/ml).

Purification of fresh human tumour cells

Malignant ascites was immediately centrifuged at 400 *g* for 5 min and then suspended in complete medium. Freshly excised tumour tissues were processed by enzymatic digestion [6]. Briefly, tumour tissues were dissected into pieces smaller than 2 mm^3 which were immersed in complete medium containing collagenase (2 mg/ml, type V-S; Sigma), hyaluronidase (10 U/ml, type IV-S; Sigma), and DNase-1 (0.4 mg/ml; Sigma). After 40 min incubation at 37°C, the cells were harvested, washed and suspended in complete medium.

The technique used for purification of autologous tumour cells has also been described [7]. Tumour cells obtained from solid tumour specimens and ascites were centrifuged on Ficoll–Hypaque (Pharmacia, specific gravity 1.077, Uppsala, Sweden) gradients at 400 *g* for 30 min in 50 ml tubes (400 *g* at the bottom of tubes). Mononuclear and tumour cells at the interface were collected, washed and suspended at 1×10^6/ml in complete medium. The cells were then layered on discontinuous gradients consisting of 10 ml of 100% and 15 ml of 75% Ficoll–Hypaque in 50-ml plastic tubes. After centrifugation at 400 *g* for 30 min (400 *g* at the bottom of tubes), a tumour cell-rich fraction was collected from the 75% interface. The tumour cell-enriched suspension was then layered onto discontinuous gradients containing 4 ml each of 25%, 15%, and 10% Percoll (Pharmacia, Uppsala) in complete medium in 15-ml plastic tubes. Centrifugation was performed at 25 *g* for 7 min (25 *g* at the bottom of tubes), and tumour cells depleted of lymphoid cells were collected from the bottom and the 25% interface, washed and suspended in complete medium at a concentration of 1×10^6/ml (Fig. 1). The cells thus prepared were primarily tumour cells, with less than 10% contamination by non-malignant cells, as judged by morphological examination using Papanicolaou staining or carcinoembryonic antigen (CEA) staining for CEA-positive tumour cells. The cells were found to be more than 90%–95% viable by the trypan blue dye exclusion test. The mean (S.D.) yield of purified tumour cells was 2.3 (0.8) $\times 10^6$, and the tumour cell count at the beginning of preparation was 13 (4.6) $\times 10^6$ [rate of yield = 17.7 (7.9%)].

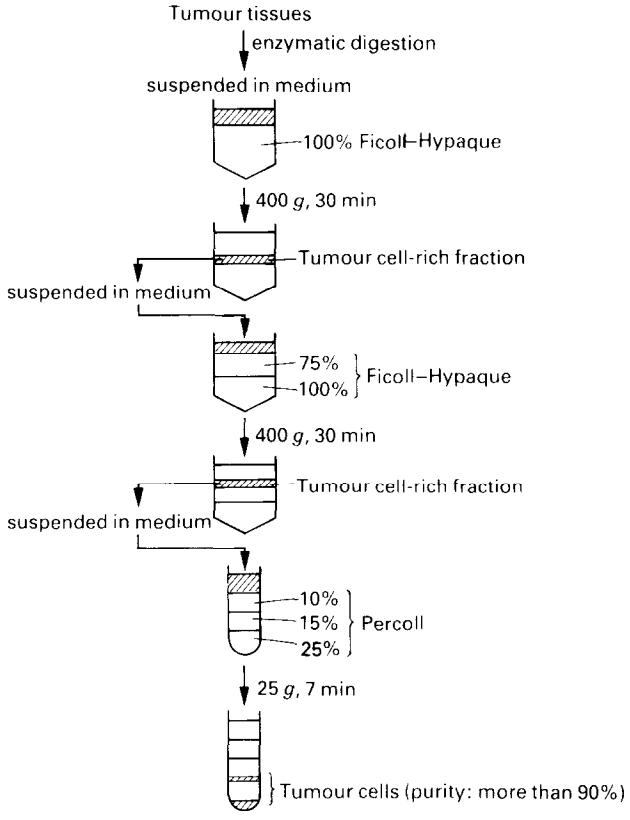

Fig. 1. Technique for the purification of tumour cells.

MTT assay

Chemosensitivity was assessed using the succinate dehydrogenase inhibition (SDI) test, using the tetrazolium salt MTT (Sigma) to measure the viability of tumour cells [1, 6]. 100 μ 1 of tumour cell suspension (5×10^5 cells/ml) was added to 25 μl of serial 10-fold dilutions of each of the antitumour drugs in 96-well flat-bottomed microtitre plates (Corning), and incubated at 37°C in a humidified 5% CO_2 atmosphere for 96 h. Each drug dilution was assessed in triplicate. Three microtitre wells containing tumour cells suspended in 125 μl of complete medium (total tumour cell number was equivalent to that in the test wells) were used as controls for cell viability, and three wells containing only complete medium were used as controls for non-specific dye reduction. After incubation, 15 μl/well of MTT solution with 10 μmol/l of sodium succinate was added to all the wells, and the plates were incubated for an additional 4 h. Acid–isopropanol (100 μl 0.04 mol/l HCl in isopropanol) was

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Table 1. Purity of tumour cells after each preparation step

Preparation step	Purity (%) Solid tumour (n = 44)	Malignant ascites (n = 24)
Enzymatic digestion or centrifugation alone	41.6 (15.3)	50.2 (25.4)
Ficoll–Hypaque discontinuous gradient	63.1 (12.9)*	65.7 (21.1)
Percoll discontinuous gradient	91.3 (4.5)*	90.4 (3.6)*

The tumour cells were enriched to a purity of 90% by the 3-step Percoll discontinuous gradient. * $P < 0.01$, compared with the purity obtained by enzymatic digestion or centifugation alone.

then added to all wells, the mixtures were pipetted, incubated at 37°C for 30 min, and again pipetted thoroughly to dissolve the dark blue crystals. The plates were then read on a microplate reader (Corona Electric, MTP-32) using a test wavelength of 570 nm and a reference wavelength of 630 nm. The control wells without tumour cells had an OD of less than 0.005.

The inhibition rate was calculated as (1 − OD drug treated/OD control) × 100.

The background of tumour cells (including dead cells) without addition of MTT had an OD of less than 0.02 after 96 h incubation; therefore the influence of dead tumour cells could be ignored in the present study.

Chemotherapy on the basis of the results of the MTT assay

31 of the 68 patients performed the MTT assay had evaluable lesions. They received cancer chemotherapy on the basis of the results of the MTT assay, and were administered drugs that the inhibition rates were more than 50% at 10 times peak plasma concentration. All subjects gave informed consent to be studied as outlined by the Ethical Committee on Human Research, Wakayama Medical College.

Statistical analysis

Significant differences were determined by paired t test or non-parametric Wilcoxon matched pairs signed-rank test, χ^2 test, and generalised Wilcoxon test for survival. P less than 0.05 was considered to be statistically significant.

Table 2. Comparison of OD_{570} obtained from tumour cells and non-malignant cells

Cases	Diagnosis	Age/sex	OD_{570} Tumour cells	Non-malignant cells
6	Malignant lymphoma	42/F	0.26	0.21
9	Oesophageal cancer	84/M	0.32	0.10
10	Oesophageal cancer	46/F	0.42	0.16
18	Gastric cancer	36/F	0.43	0.25
37	Ovarian cancer	54/F	0.20	0.22
38	Ovarian cancer	17/F	0.22	0.13
53	Pancreatic cancer	63/M	0.45	0.20
55	Gastric cancer	64/M	0.27	0.14
58	Gastric cancer	65/F	0.36	0.23
61	Gastric cancer	78/M	0.40	0.26
	Mean (S.D.)		0.33 (0.09)*	0.19 (0.05)

OD_{570} obtained from tumour cells was higher than that from non-malignant cells (*$P < 0.01$).

Table 3. Comparison of purified tumour cell and non-malignant cell sensitivity

Cases	Diagnosis and source of MTT assay	Inhibition rate (%) by anticancer agents C	E	M	D	5-FU
6	Malignant lymphoma					
	Tumour cells	74.5	0	46.4	80.9	52.0*
	Non-malignant cells	70.6	27.7*	65.5*	80.1	41.6
9	Oesophageal cancer					
	Tumour cells	89.0	76.5*	70.1	71.6*	64.9*
	Non-malignant cells	100.0*	30.0	85.6*	60.0	27.8
10	Oesophageal cancer					
	Tumour cells	53.9	37.5	53.0	68.0	82.6
	Non-malignant cells	47.0	28.2	60.8	58.2	80.4
18	Gastric cancer					
	Tumour cells	75.0*	67.5*	91.2*	0	78.7*
	Non-malignant cells	61.1	5.6	41.7	26.1*	11.2
37	Ovarian cancer					
	Tumour cells	17.0	N.T.	0	20.8	21.3
	Non-malignant cells	19.6	N.T.	0	45.6*	36.7*
38	Ovarian cancer					
	Tumour cells	62.1	89.8*	N.T.	56.4*	N.T.
	Non-malignant cells	52.5	10.0	N.T.	39.7	N.T.
53	Pancreatic cancer					
	Tumour cells	21.3*	15.1	34.5	58.8*	61.8*
	Non-malignant cells	10.5	35.2*	40.1	25.9	39.8
55	Gastric cancer					
	Tumour cells	25.2	41.9	83.3*	84.1*	74.8*
	Non-malignant cells	30.8	53.2*	70.1	65.8	59.4
58	Gastric cancer					
	Tumour cells	91.7*	58.0	87.6*	89.1*	59.5*
	Non-malignant cells	53.2	78.5*	60.3	67.1	30.7
61	Gastric cancer					
	Tumour cells	59.1*	63.8*	7.9	59.3*	0
	Non-malignant cells	31.5	50.3	0	21.2	0

Anticancer agents: C_{max} × 10.
*Differences greater than 10% between the inhibition rates of tumour and non-malignant cells.
C = cisplatin, E = etoposide, M = mitomycin, D = doxorubicin.

RESULTS

Purity of fresh human tumour cells

The purity (S.D.) of tumour cells immediately after enzymatic digestion alone for solid tumours or centrifugation alone for malignant ascites was 41.6 (15.3)%, and 50.2 (25.4)%, respectively. After processing on the Ficoll–Hypaque discontinuous gradients the purity increased to 63.1 (12.9)% and 65.7 (21.1)%, respectively. Tumour cells in both solid tumour and malignant ascites samples were enriched to 90% using the Percoll discontinuous gradients ($P < 0.01$ by paired t test) (Table 1).

Further, this purification method could be performed for all types of tumours, and the percentage of tumour cells after 96 h incubation was equivalent to that before incubation by morphological examination [from solid tumour: 92.6 (5.7)%, from peritoneal effusion: 91.3 (6.2)%], and the viability of tumour cells after 96 h incubation was more than 90% in control wells (tumour cells in complete medium alone).

OD_{570} from tumour cells and non-malignant cells

The comparison of OD_{570} between isolated tumour cells and non-malignant cells in tumour tissue was performed in 10 patients whose tumour cell counts and non-malignant cell counts were sufficient for MTT assay. The other patients, except for those in Tables 2 and 4, were assessed by chemosensitivity testing of tumour cells alone. After 96 h incubation, the OD_{570} from tumour cells was 0.33 (0.09), and that from non-malignant cells 0.19 (0.05) ($P < 0.01$ by Wilcoxon test) (Table 2).

Comparison of chemosensitivity between tumour cells and non-malignant cells

The results of chemosensitivity comparisons are shown in Table 3. The purity of tumour cells was greater than 90.0% in all cases. In cases 18 and 55, all inhibition rates, except that by doxorubicin in case 18 and that by etoposide in case 55, for tumour cells were higher than those obtained for non-malignant cells. In case 10, the inhibition rates of tumour cells and non-malignant cells were equivalent. However, non-malignant cells were more sensitive than tumour cells to 5-FU and doxorubicin in case 37. The inhibition rates of tumour cells and non-malignant cells by anticancer agents differed in each case; however, the inhibition rates for tumour cells were 10% greater than those for non-malignant cells in 5, 4, 3, 6 and 6 of the 10 cases against cisplatin, etoposide, mytomycin, doxorubicin and 5-FU, respectively.

Comparison of the chemosensitivity of purified tumour cells and crude cells just separated from tumour tissues

We compared the inhibition rates of purified tumour cells and crude cells just separated from tumour tissues in specimens from 5 patients. In case 60, the inhibition rates of purified tumour cells by anticancer agents were greater than those of unseparated cells, except for etoposide; however, in case 63, these rates were less than those for unseparated cells, except for doxorubicin. The inhibition rates for purified tumour cells were 10% greater than those for unseparated cells in 4, 2, 2, 1 and 2 of the 5 cases inhibited by cisplatin, etoposide, mitomycin, doxorubicin and 5-FU, respectively (Table 4).

Table 4. Chemosensitivities of purified tumour cells and crude cells just separated from tumour tissues

Cases	Diagnosis and source of MTT	Inhibition rates (%) by anticancer agents				
		C	E	M	D	5-FU
57	Gastric cancer					
	Tumour cells	51.3*	43.6*	15.5	4.6	0
	Unseparated cells	3.9	21.0	16.5	12.2	0
59	Gall bladder cancer					
	Tumour cells	56.7*	10.5	50.5*	21.5	10.3
	Unseparated cells	22.1	14.6	35.0	22.3	0
60	Gastric cancer					
	Tumour cells	65.5*	33.6	44.4*	58.5*	43.4*
	Unseparated cells	50.0	29.6	10.7	42.1	10.8
63	Gastric cancer					
	Tumour cells	90.0	43.7	85.0	98.7	56.3
	Unseparated cells	100.0*	74.0*	100.0*	100.0	93.2*
66	Gastric cancer					
	Tumour cells	94.5*	71.3*	92.1	83.7	80.2
	Unseparated cells	46.7	46.7	91.2	85.8	74.1

*Differences greater than 10% between inhibition rates of purified tumour cells and unseparated cells.
C = cisplatin, E = etoposide, M = mitomycin, D = doxorubicin.

Table 5. Chemosensitivity of purified tumour cells separated from various cancers

Diagnosis (n)	Positive sensitivity (%)				
	C	E	M	D	5-FU
Gastric cancer (29)	62.1†	20.7	48.3	37.9	48.3
Ovarian cancer (8)	37.5	25.0	25.0	25.0	25.0
Malignant lymphoma (7)	57.1	57.1	28.6	71.4	28.6
Colon cancer (7)	57.1	28.6	85.7*	57.1	57.1
Pancreatic cancer (6)	33.3	33.3	33.3	50.0	50.0
Oesophageal cancer (5)	80.0	40.0	60.0	60.0	80.0

Positive sensitivity indicates an inhibition rate greater than 50% at 10 times peak plasma concentration. In gastric cancer, there was a significant difference between positive sensitivity rates of cisplatin and etoposide (†$P < 0.01$ by χ^2 test) and in colon cancer a significant difference between these rates of mitomycin and etoposide (*$P < 0.05$).
C = cisplatin, E = etoposide, M = mitomycin, D = doxorubicin.

Chemosensitivity of purified tumour cells separated from various cancers

The rates of high sensitivity (i.e. inhibition rates of more than 50% at 10 times peak plasma concentration) of the anticancer agents are shown in Table 5. In gastric cancer, cisplatin was a more active drug than etoposide ($P < 0.01$) and in colon cancer mitomycin was more active than etoposide ($P < 0.05$). However, there were no significant differences in the high sensitivity rates of any drugs among the various types of cancer.

Clinical correlation

Of the 68 patients, 31 had evaluable lesions, and they received cancer chemotherapy according to the results of the MTT assay using highly purified tumour cells. Clinical responses were obtained in 10 of the 31 patients (response rate: 32.3%). 5 patients including 3 with gastric cancer and 2 with malignant lymphoma, achieved complete response (CR), and 5 patients including 3 with gastric cancer, 1 with oesophageal cancer, and 1 with pancreatic cancer had a partial response (PR) (Table 6).

DISCUSSION

The SDI test using tetrazolium salt MTT is a rapid and semi-automated quantitative assay for screening antitumour drugs [8]. The SD activity correlates with cell viability, and therefore seems to be useful for clinical application. However, the contamination by non-malignant cells in tumour tissues may influence the results of this assay. In leukaemia, the purity of cells in peripheral blood or bone marrow is fairly high, so that centrifugation with 100% Ficoll–Hypaque alone gave a concentration of greater than 90% blast cells for use in the MTT assay [9–11]. On the other hand, the MTT assay has been performed without purification of tumour cells from samples of solid tumour tissues [4, 5].

This study is the first to report the purification of tumour cells from solid tumours as a source for the MTT assay, and that the sensitivity of tumour cells was distinct from that of the non-malignant cells contaminating in tumour tissues. Enrichment of tumour cells leads to a loss of stromal cells, but these

Table 6. Clinical response according to MTT assay using purified tumour cells

Case	Age/sex	Cancer	Stage	Evaluable lesions	Chemotherapy	Response	Survival (mo)
33	39/F	Gastric	IV	Malignant ascites	C, E, M	CR	9.5
51	55/F	Gastric	IV	Malignant ascites	M, D	CR	4.8 (alive)
57	67/F	Gastric	IV	Lymph nodes	C, E	CR	4.0 (alive)
4	55/M	ML	III	Lymph nodes	D, E, F	CR	28.0 (alive)
6	42/F	ML	IV	Lymph nodes	C, D, F	CR	9.6
39	54/M	Oesophageal	IV	Lung metastasis	C, F	PR	5.5
12	56/M	Gastric	IV	Malignant ascites	M, F	PR	13.5
5	39/F	Gastric	IV	Malignant ascites	C, M	PR	12.0
40	66/F	Gastric	IV	Malignant ascites	C, M	PR	5.0
53	63/M	Pancreatic	IV	Malignant ascites	F, D	PR	6.5

ML = malignant lymphoma, C = cisplatin, E = etoposide, M = mitomycin, D = doxorubicin, F = 5-FU. Cisplatin and mitomycin were administered intraperitoneally for malignant ascites, and other drugs intravenously.

were not necessary for maintaining the viability of tumour cells during culture; and moreover the sensitivity of the purified tumour cells was also distinct from that of crude cells just separated from tumour tissues. Therefore, it can be seen that tumour cells should be purified using the various gradient techniques for the MTT assay.

In the present study, various kinds of tumour tissues were separated to provide purified tumour cells, and this purification method could be performed in all tumour entities. The background of tumour cells, including dead cells, without the addition of MTT was minimal and could be ignored in purified materials from solid tumours for MTT assay. It has been reported that tumour tissues are more sensitive to various antitumour drugs than adjacent normal mucosal tissues. However, some patients showed less or equal sensitivity of tumour tissues to antitumour drugs compared with that of normal mucosal tissue [12]. However, these workers did not compare the chemosensitivity of purified tumour cells and normal mucosal cells, and that of tumour cells and non-malignant cells contaminating in tumour tissue; they only compared the chemosensitivity of crude cells just separated from tumour tissues and normal mucosal cells. Our results in the present study show that the OD_{570} of purified tumour cells was greater than that of non-malignant cells contaminating tumour tissues, and that the chemosensitivity for tumour cells and non-malignant cells differed in each case. Moreover, the inhibition rates of tumour cells by various drugs in gastric cancer were greater than those of non-malignant cells, as compared with other tumours.

Comparison of chemosensitivity (Table 3) showed that purified tumour cells were more sensitive than non-malignant cells in patients with gastric cancer, and Table 5 shows that the sensitivity to cisplatin was greater than that to etoposide in gastric cancer and the sensitivity to mitomycin was greater than that to etoposide in colon cancer. Therefore, gastric cancer is more sensitive to cisplatin, and colon cancer more sensitive to mitomycin than the other tumour types are.

Using the procedure described by Mosmann with acid isopropyl alcohol as solvent [1], we were sometimes unable to achieve complete solubilisation of the formazan crystals, and modified the technique so that after pipetting, specimens were incubated at 37°C for 30 min, and then pipetted again thoroughly to dissolve the dark blue crystals. Also, we have recently used dimethyl sulphoxide (DMSO) as a solvent [13].

Clonogenic assays have been applied extensively in the assessment of chemosensitivity testing [14]. However, they have been reported to have several problems, including low plating efficiency and prolonged time requirements. Von Hoff *et al.* reported that the plating efficiency in a clonogenic assay was only 41% [15], but the successful rate of MTT assay in the present study was 87.2%. The drug concentrations used in the present study were 10 times peak plasma concentration. Serial 10-fold dilutions were performed and sensitivity was assessed at × 10, × 1, and × 1/10 concentrations. However, the adequate numbers of purified tumour cells to assess the chemosensitivity at three concentrations (× 10, × 1, × 1/10) could be separated only in 13 of the 68 patients, and succinate dehydrogenase activity of drug-treated cells was reduced to below 50% of that in control cells in only 34% of patients at × 1 concentration and in 19% of patients at × 1/10 concentration (unpublished data). Carmichael *et al.* demonstrated that good correlation was observed between the dose-response curves of clonogenic and MTT assays [13]. In solid tumours, the concentration of drugs used in MTT assay has usually been at 10 times peak plasma concentration [4, 5, 12] or within a clinically achievable range [16]. An *in vivo* subrenal capsule (SRC) assay has also been developed in response to the need for a rapid *in vivo* test system for drug screening, and it was found to have a better prediction of the clinical response [17, 18]. A positive correlation was noted between the SRC assay and the MTT assay performed at 10 times peak plasma concentration [19].

We therefore used the 10 times peak concentrations to determine the administered drugs in clinical trial. We are now proceeding to investigate, by prospective study, whether the results of MTT assay using highly purified fresh human tumour cells correlate with clinical response and further which drug concentrations should be used in the MTT assay.

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Doxorubicin Interactions at the Membrane: Evidence for a Biphasic Modulation of Inositol Lipid Metabolism

Michael G. Thompson and John A. Hickman

Doxorubicin, when incubated for 30 minutes with [^{32}P]-labelled human erythrocyte membrane vesicles, produced an elevation of [^{32}P]inositol-1,4,5-trisphosphate levels. The maximum rise was obtained with 10^{-8} mol/l doxorubicin [132 (S.E. 13%) of control, $n = 6$, $P = 0.001$]. However, when the inositol lipids were examined, there was no evidence that doxorubicin stimulated the breakdown of [^{32}P]phosphatidylinositol-4,5-bisphosphate under resting conditions, suggesting that the elevated levels of [^{32}P]inositol 1,4,5-trisphosphate were not the result of the stimulation of phospholipase C. Instead, it was found that the dephosphorylation of inositol 1,4,5-trisphosphate by a 5′-phosphomonoesterase was partially inhibited by 10^{-8} mol/l doxorubicin so that the rise in [^{32}P]inositol 1,4,5-trisphosphate resulted from the inhibition of the breakdown of constitutively released [^{32}P] inositol 1,4,5-trisphosphate. Similar data was also obtained with another aminoglycoside antibiotic, neomycin. The release of [^{32}P] inositol 1,4,-bisphosphate and [^{32}P] inositol 1,4,5-trisphosphate and the breakdown of the inositol lipids in response to calcium (2.5×10^{-4} and 10^{-3} mol/l) stimulation was enhanced by doxorubicin (10^{-6} to 10^{-12} mol/l). These effects on resting and stimulated inositol lipid metabolism are discussed with reference to the paradoxical effects of doxorubicin to both stimulate and inhibit proliferation, according to concentration.
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INTRODUCTION

THE AMINOGLYCOSIDE antibiotic doxorubicin is a potent antitumour drug in humans yet its mechanism of action remains controversial. As an amphipath it is capable of physical interactions with DNA, the nuclear enzyme topoisomerase II and membranes [1–3]. Evidence that its cytotoxicity is expressed through a membrane-mediated effect comes from both biophysical and biochemical studies [4–6] but few reports have attempted to address the question of what consequences may ensue from a doxorubicin–membrane interaction which could account for cytotoxicity. Recently, Poseda *et al.* have suggested an attractive but as yet unsubstantiated hypothesis that a doxorubicin-mediated elevation of diacylglycerol, brought about by membrane receptor activation, could activate protein kinase C [7]. This idea implies that doxorubicin becomes enmeshed in growth factor-mediated events in the cell and it might therefore be expected that this would modulate the ability of the cells to traverse the G_1–S transition of the cell cycle. Most studies do