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Short communication

Influence of clonogenic assay methodology on measurement of drug sensitivities in vitro

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Summary. To assess the influence of clonogenic assay methodology on measurement of reproductive cell kill, the in vitro sensitivities of a human bladder cancer cell line, RT112, to methotrexate and adriamycin were determined using ten different procedures. Marked differences in dose-response to methotrexate, but not adriamycin, were observed.

Introduction

Clonogenic assay is the accepted technique for measuring reproductive cell survival of cell lines following exposure to chemotherapeutic drugs [4], but the methodology is not standardised. Cells may be left in situ or transferred immediately or at some time after drug exposure, and some lines require a feeder layer to permit colony development. These assay modifications influence reproductive cell survival [5], but few comparative experimental data are available. The purpose of this study was to compare measurements of in vitro drug sensitivities using ten variations of the clonogenic assay procedure.

Material and methods

RT112 cells were grown in RPMI 1640 medium (Gibco, Paisley, Scotland) containing 2m*M* L-glutamine (Gibco) and 5% heat-inactivated foetal bovine serum (Flow, Irvine, Scotland) at 36.5 °C in a humidified atmosphere of 5% CO_2 in air. A single batch of serum was used throughout. For the in situ assay 1000 exponentially growing cells were plated in 5 cm plastic petri dishes (Gibco) containing 5 ml medium and incubated for 48 h before drug exposure. For the transfer assay 1000 (low density) or 12 800 (high density) exponentially growing cells were plated in microtest plate wells containing 0.2 ml medium and incubated for 48 h before drug exposure. Methotrexate (MTX) (Lederle, Gosport, England) and adriamycin (ADR) (Farmitalia Carlo Erba, St. Albans, England) were dissolved in PBSA immediately before use and serially diluted in medium. Following a 24 h drug exposure cells were washed 3 × with medium and once with PBSA. For the in situ assay the medium was replenished. For transfer assay the cells were detached either immediately or 24 h later using 0.2 ml of 0.01% trypsin (Difco, London, England 1:250) in 0.003% versene and diluted to produce 100–200 colonies per 5 cm dish. To determine the effect of feeder layers 20 000 Swiss 3T3 K mouse fibroblasts were plated per 5 cm dish. After 10–14 days the medium was replenished and RT112 cells plated onto the monolayer, prior to drug exposure for the in situ assay or after drug exposure for the transfer assay. For all assay systems the medium was replenished at 7 days and the colonies fixed and stained with 10% Giemsa after 14 days. The mean colony-forming ability was expressed as a percentage of that of controls. Five control and three drug-treated replicates were used at each drug concentration. Data for each of the ten assay procedures were derived from a minimum of three experiments.

To measure tritiated thymidine labelling indices exponentially growing cells were plated in 5 cm petri dishes at cell numbers equivalent to those used for the clonogenic assay procedures, i.e., 1000, 61 000, and 780 000 for the in situ, low- and high-density transfer assays, respectively. Following a 48 h incubation fresh medium containing 1 μCi/ml [methyl-^{3}H]thymidine (Amersham, Buckinghamshire, England; 25 Ci/mmol) was added and after exposure periods of 15 min and 2, 4, 8, 24, 48, 72 and 96 h duplicate dishes were processed and autoradiography was carried out as previously described [2].

Results

The dose-response curves to MTX and ADR are shown in Fig. 1. Clonogenic cell survival was markedly influenced by the methodology used following exposure to MTX, but not after exposure to ADR. In general, cells treated and left in situ were less sensitive than those transferred, and in transfer assays the cells were less sensitive and the ID70s (drug concentration reducing clonogenic survival by 70%) were more variable at the lower density.

Exponential/plateau dose-response curves for MTX were obtained with cells plated at the highest density and transferred immediately onto plastic or feeder layers (Fig. 1 c). Using other assay methods, however, the plateau region was less sharply defined. Exponential dose-response curves were obtained for ADR with all procedures. However, a more pronounced shoulder region was obtained with in situ assays and for cells plated at the lower density. No consistent differences were observed as a result of a 24 h delay between drug exposure and cell transfer or with feeder layers with either drug.

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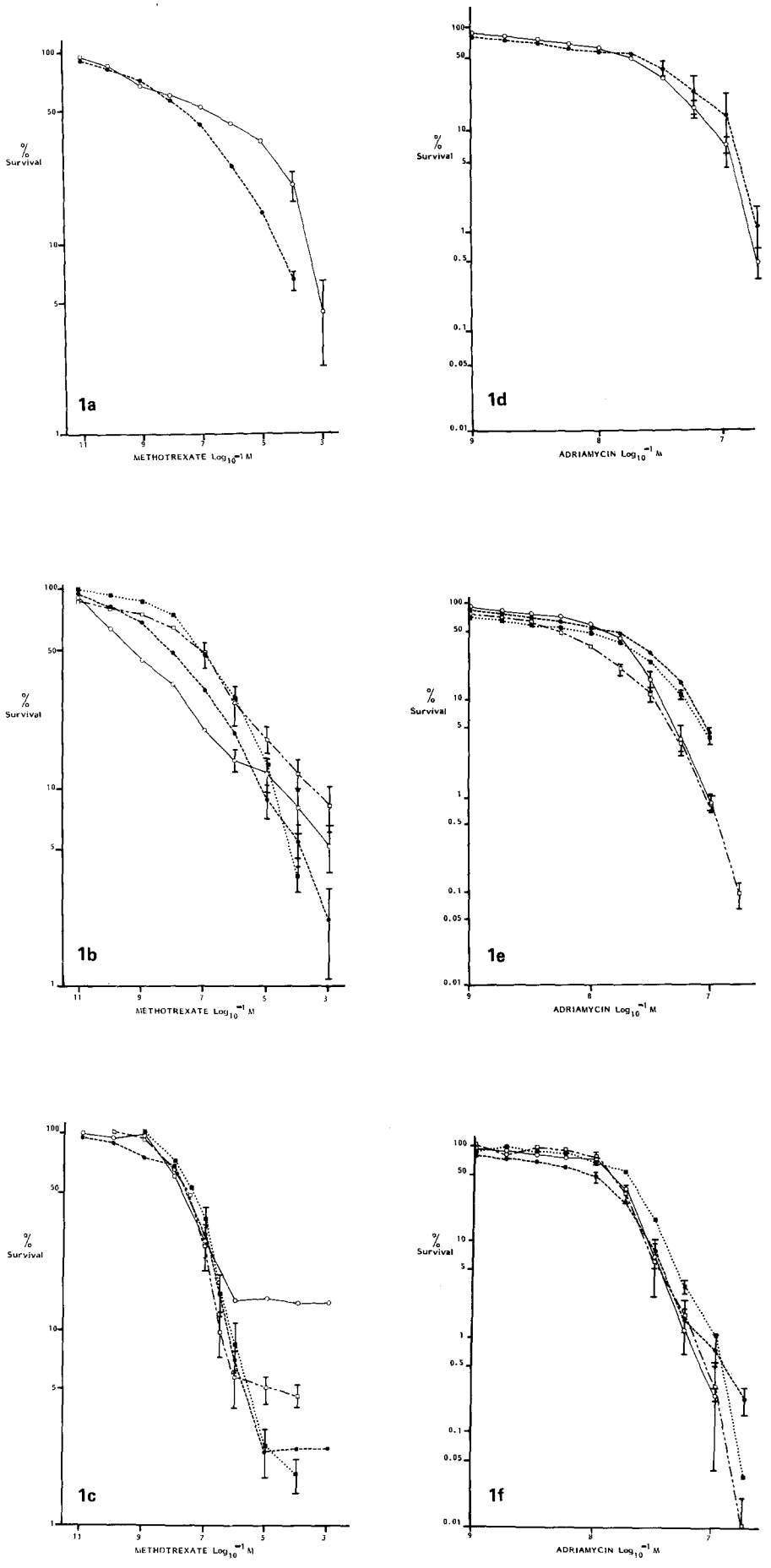

Fig. 1a–f. Comparison of dose response curves obtained using different clonogenic assay procedures after a 24 h exposure to methotrexate or adriamycin. **a, d** In situ assay: ● with 3T3 feeder cells; ○ without feeder cells. **b, e** low-density cells (1000/well during drug exposure); ○ immediate transfer; □, transfer after 24 h delay; ●, immediate transfer onto 3T3 feeder cells; ■, transfer onto 3T3 feeder cells after 24 h delay. **c, f** High density cells (12 800/well during drug exposure); ○, immediate transfer; □, transfer after 24 h delay; ●, immediate transfer onto 3T3 feeder cells; ■, transfer onto 3T3 feeder cells after 24 h delay. Each point represents the mean of at least three experiments, and only standard error bars in excess of 10% are included

Table 1. The labelling indices of cells plated at densities of 1000, 61,000 and 780,000 per 5 cm dish, equivalent to the cell concentrations used for the in situ, low and high cell density transfer assays respectively, and exposed to ^{3}H-thymidine continuously for periods ranging from 15 min to 96 h

Exposure to ^{3}HTdR (h)	Percentage of cells labelled		
	In situ	Low density transfer	High density transfer
0.25	9.9	12.3	20.4
2	16.5	28.1	35.3
4	21.8	32.6	41.6
8	32.1	40.6	52.2
24	95.4	97.6	100.0
48	100.0	100.0	100.0
72	100.0	100.0	100.0
96	100.0	100.0	100.0

The ^{3}H-thymidine labelling indices of the three cell concentrations are shown in Table 1. Within 2 h 35% of cells plated at high density were labelled, but 5 and 9 h elapsed before a similar proportion of cells plated at concentrations equivalent to the low cell density and in situ assays, respectively, were labelled. Nevertheless, within 24 h, at least 95% of the cells were labelled at each density, and after 48, 72, and 96 h of continuous exposure no unlabelled cells were observed.

Discussion

Clonogenic assay procedures for monolayer cultures are of two types: cells are either left in situ or transferred following drug exposure. Using the in situ assay low cell numbers must be plated for discrete colonies to be obtained. It was postulated that differences in results obtained using these two methods might be related to cell density, and therefore the sensitivities of cells plated at low and high density were also compared by means of the transfer assay. The responses of cells plated at the lower density had features in common with those obtained using the in situ assay, including a pronounced shoulder at low ADR concentrations and a less obvious plateau at high MTX concentrations. Cell density also influenced the tritiated thymidine labelling index. As MTX may only kill cells in S phase [3], these cell kinetic data may explain why much greater differences in survival were observed with MTX than with ADR.

Delay between drug treatment and cell transfer may permit some clonogenic cells to recover from potentially lethal damage [1]. In this study, however, a 24 h delay had little effect on survival following drug exposure. In this study, however, a 24 h delay had little effect on survival following drug exposure. The addition of feeder cells may also influence measurements of in vitro drug sensitivity, for instance by metabolic co-operation. RT112 cells do not show a 'feeder-effect', and 3T3 feeder layers had little influence on the dose-response curves.

This study shows that clonogenic assay procedure can influence the measurement of in vitro drug sensitivities, and indicates that standardisation is essential if comparative studies are to be of value.

References

1. Barranco SC, Bolton WE, Novak JK (1980) Time-dependent changes in drug sensitivity expressed by mammalian cells after exposure to trypsin. NCI 64: 913–916
2. Brooks RF, Riddle PN, Richmond FN, Marsden J (1983) The Gl distribution of "Gl-less" V79 Chinese hamster cells. Exp Cell Res 148: 127–142
3. Drewinko B, Barlogie B (1964) Cell cycle perturbation effects: In: Fox BW, Fox M (eds) Antitumor drug resistance. Springer, Berlin Heidelberg New York, pp 101–141
4. Hill BT (1983) The use of continuous human tumour cell lines to evaluate effects of drugs by clonogenic assays: In: Dendy PP, Hill BT (eds) Human tumour drug sensitivity testing in vitro – techniques and clinical applications. Academic Press, London, pp 129–145
5. Twentyman PR (1979) Timing of assays: An important consideration in the determination of clonogenic cell survival both in vitro and in vivo. Int J Radiat Oncol Biol Phys 5: 1213–1220

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