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The Growth and Cellular Kinetics of Human Cervical Cancer Spheroids in Relation to Drug Response

M.O. AFFIA,* C.A.F. JOSLIN, R.I. ROTHWELL and C.D. BOOTHBY*

University Department of Radiotherapy, Tunbridge Building, Cookridge Hospital, Leeds LS16 6QB, U.K.

Abstract—*A methodology for culturing multicellular spheroids directly from tumour biopsy material has been established with a success rate of 82% for cervical carcinomas. Spheroids, selected for uniform size and shape, have been subjected to quantitative assay to determine their population dynamics using ^{3}H-Tdr autoradiography and have also been treated with 13 commonly used anti-cancer drugs. Several characteristics of cervical spheroids, e.g. growth rate, labelling index, thickness of viable cell rim, were similar for drug responding and non-responding spheroids but the faster growing spheroids responded to an increased number of types of drugs. Spheroids derived from poorly differentiated tumours also showed an increased response to drugs compared to those from moderate or well differentiated tumours.*

INTRODUCTION

SEVERAL studies have demonstrated a degree of correlation between drug sensitivity *in vitro* and *in vivo* tumour response [1–3]. A reason why the accurate prediction of clinical response to cancer chemotherapy has yet to be achieved [4] is the lack of model systems that adequately reflect the complexity of the *in situ* tumour environment. Apart from the inherent chemosensitivity of cancer cells, the biology of solid tumours is known to be important in the determination of therapeutic response [5, 6]. An *in vitro* model of tumour growth where the heterogeneity of cellular oxygen status and proliferative state is analagous to that found in a solid tumour *in vivo* is the multicellular spheroid described by Sutherland, McCredie and Inch in 1971 [7].

We have established a procedure to culture multicellular spheroids directly from patient biopsy material of bronchial, colorectal, breast and cervical carcinomas [8–11]. Spheroids of a suitable size (250–300 μm) for *in vitro* chemotherapy testing, or irradiaton experiments are available within 4–8 days from the start of the procedure.

We report here on the methodology employed in the culture of multicellular spheroids from cervical tumour biopsy samples, and studies on their growth kinetics. The procedures involved in the use of multicellular spheroids as a model system for the *in vitro* testing of cytotoxic drugs are also reported.

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MATERIALS AND METHODS

Pre-treatment cervical tumour biopsies were cleaned of haemorrhagic and mucinous debris, minced, drawn up several times through the free end of a 1 ml syringe and stirred in 0.25% trypsin in Hanks balanced salt solution (HBSS) for 25 min at 37° C. The resulting cell suspension was separated by passing through a wire gauze to exclude undigested tissue. The filtrate was centrifuged at 170 ***g*** for 8 min and the cell pellet re-suspended in complete medium [Medium 199, 15% Newborn Calf serum, 1 mM Glutamide, 1.7 g/l sodium bicarbonate, 20 mM Hepes, non-essential amino acids (NEAA) (all obtained from Gibco Biocult)], plus gentamycin. Cells were counted in an improved Neubauer haemocytometer and their viability determined by Trypan blue (Flows Labs) exclusion.

Our method for initiating spheroids is as follows: 2×10^5 cells were inoculated into tissue culture flasks (25 cm^3, Sterilin) base coated with 1% agar in complete medium, and 10 ml complete medium added. The cells were allowed to settle on the agar bottom and by gentle horizontal agitation of the flasks (from side to side) dense bands of tumour cells were then produced. The flasks were left for about 24 hr in a warm room (37° C) to facilitate cell adhesion. The dense bands of cells

were reduced to smaller aggregates by more vigorous agitation and a further 5 ml fresh complete medium added. Twenty-four hours later the cell aggregates were transferred to larger agar bottom-coated flasks (75 cm^3, Sterilin) and 20 ml complete medium added. When the tumour spheroids appeared firm enough to withstand stirring, they were transferred into 700 ml spinner flasks (Techne, Cambridge) containing 500 ml complete medium for further growth. The medium was changed three times a week. At the time of change, 20 spheroids were measured (mean of two diameters at right angles) using an inverted microscope with a calibrated eyepiece, and the mean diameter of the 20 spheroids calculated.

For pulse labelling, pre-warmed medium containing 1 μCi/ml tritiated thymidine, (^{3}H-Tdr) (5 Ci/mmol; Radiochemical Centre, Amersham) was added to the spheroids and the flasks incubated at 37° C for 1 hr. Immediately after incubation the spheroids were washed with HBSS, fixed in Bouin's solution and stored in 70% ethanol at 4° C.

For continuous labelling, spheroids were incubated with complete medium containing 0.1 μCi/ml ^{3}H-Tdr. During the next 48 hr spheroids were periodically removed from flasks, washed, fixed and stored.

For histological examination, spheroids were washed in HBSS, fixed for 1.5 hr in Bouin's solution, and removed to 70% ethanol at 4° C for storage. The spheroids were embedded in 1% alginate drops to facilitate further handling. After dehydration in a graded alcohol series (70–100%) and clearing in xylene, each alginate ball containing up to five spheroids was vacuum-embedded in Ralwax (R.A. Lamb, London), mounted in a paraffin block and serial 5 μm sections cut through the spheroids. These were placed on glass slides, dewaxed and stained with haematoxylin and eosin (H and E).

^{3}H-Tdr labelled spheroids were processed as for histological examination and the sections prepared for autoradiography by the dipping method [12].

The mitotic index (MI) was determined by mitotic cells/total cells. The ^{3}H-Tdr labelling index (LI) was determined by labelled cells/total cells. The growth fraction (GF) following continuous labelling was determined by a percentage labelled cells/percentage labelled mitosis [13].

For drug assay: 20 spheroids, of uniform spherical shape, and as near uniform diameter as possible were selected for each measurement. Generally, these were within the range 250–300 μm, but for certain experiments spheroids were selected close to 500 μm. Spheroids were then washed in HBSS, and treated with a 1 hr exposure daily for 5 consecutive days to the clinically achievable peak plasma drug concentrations [14]. This was 0.08 μg/ml for actinomycin-*D* (Act-D), 0.6 μg/ml for adriamycin (Adr), 0.06 μg/ml for cytosine arabinose (Ara-C), 1 μg/ml for 1,3-bis (2-chloroethyl)-1-nitrosourea (BCNU), 2.5 μg/ml for bleomycin (Bleo), 2 μg/ml for cisplatinum (Cispl), 60 μg/ml for 5-fluorouracil (5-FU), 2.5 μg/ml for melphalan (Melph), 3 μg/ml for methotrexate (Meth), 1 μg/ml for mitocycin-*C* (mit-c), 5 μg/ml for vinblastine (Vinb), 1 μg/ml for Vincristine (Vinc) and 1 μg/ml for methyl-CCNU (MeCCNU).

Measurements were carried out in the same manner as described for the growth period. Regression was calculated as a percent reduction in the mean diameter of the 20 spheroids based on the mean diameter on completion of exposure to the cytotoxic drug.

RESULTS

Adequate numbers of spheroids for the full series of chemotherapy tests (13 agents), of size exceeding 250 μm, were cultured from tumour tissue biopsies from 46 patients (55% of biopsies). Spheroids were successfully cultured from a further 22 patients (27% of biopsies), some exceeding 600μm in diameter. However the numbers of spheroids were inadequate for a full series of chemotherapy measurements, and these data have not therefore been included. Numbers of spheroids available did not, however, allow measurements of the effects of all the cytotoxic drugs, which we wished to screen initially, to be carried out more than once. The chemotherapy data presented, therefore, are the results of single experiments.

For the majority of the cervical tissue biopsies the number of spheroids produced per 2×10^5 viable cells have ranged from 63 to 519. The spheroids attained a diameter of about 100 μm within 2–3 days of culture, followed by an approximate exponential growth phase and finally by a slowing of growth (e.g. Fig. 1). The growth rate of spheroids from different patients ranged from 12 to 83 μm/day (at 200 μm diameter) and from 3 to 34 μm/day (at 600 μm diameter).

Central necrosis was not observed in spheroids less than 200 μm diameter. However, beyond 300 μm diameter zones of viable cells and necrosis could usually be distinguished (e.g. Fig. 2). For most spheroids the thickness of the viable rim ranged from 62 to 161 μm, but in one patient a single layer of cells lined the spheroids (Fig. 3) while for some patients central necrosis was not observed even when the spheroids had grown beyond 600 μm diameter (e.g. Fig. 4).

The labelling index (LI) of spheroids 200 μm diameter ranged from 9.0 to 31.4% and from 3.7 to 23.6% at 600 μm diameter. For most spheroids

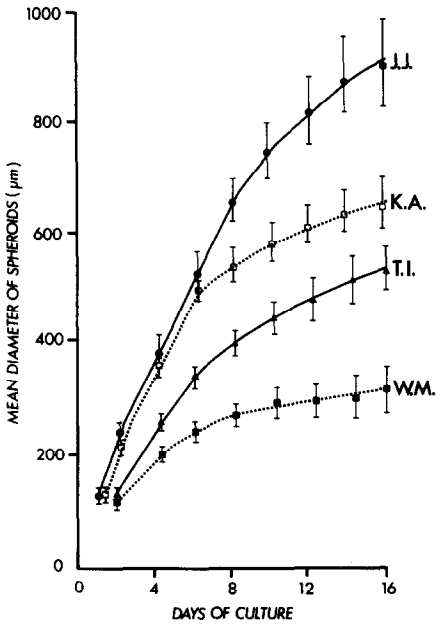


Fig. 1. Four examples selected to illustrate minimum and maximum growth rates of spheroids cultured from cervical tumour tissue samples. Each point mean of measurement of 20 spheriods ± standard error of mean (S.E.).

the LI decreased during spheroid growth (Table 1, Fig. 5b). There was no apparent correlation between the growth kinetics of cervical spheroids and the histological grading or clinical staging of the tumour from which they were derived (Table 1). The spheroids growth rate (labelling index) and thickness of viable cell rim were related (Fig. 5a). Faster growing spheroids with high LI had thicker rims of viable cells than their slower growing counterparts (Fig. 5a).

For the chemotherapy assays 20 spheroids 250–300 μm in diameter, as uniform in size and spherical shape as possible were selected. However reproducibility of measurement for 20 selected spheroids was found to be ± 7% due to small departures from uniformity of size and true spherical shape. Therefore the results of the chemotherapy assays have been presented as: less than 10% regression (reduction of diameter) was not considered significant. In the second group, a response of 10–20% regression possibly represents the loss of 1–2 cell layers from the viable rim (of 250–300 μm spheroids), was classified as a partial response. The third group with greater than 20% regression represents the loss of greater than two cell layers from the viable rim (Table 2), and therefore a significant response.

In chemotherapy assays from 46 spheroid cultures, 13 (28%) responded (> 20% spheroid regression) to three or more drugs, 10 (22%) responded to two drugs, 12 (26%) responded to only one drug (Table 3). No significant regression was produced by any of the 13 drugs used in 11 (24%) of the specimens. Seventeen spheroids derived from 17 poorly differentiated tumours responded (> 20% spheroid regression) to at least one drug, while seven from 21 moderately differentiated tumours and from four from eight well differentiated tumours did not respond (< 10% spheroid regression) to any of the 13 drugs assayed. The clinical staging of the tumour does not appear to be significantly related to the *in vitro* drug response of the spheroids produced (Table 3). As an example of the variable response to a single drug, bleomycin, Fig. 6 depicts the extent of regression observed in spheroids cultured from biopsies from 22 patients.

However due to the variable degree of penetration of different cytotoxic drugs, and variable

Table 1. Histological differentiation and clinical staging of cervical tumours, and the growth rate labelling index and thickness of the viable rim of the spheroids derived

Growth Rate μm/day at		Labelling index				
200 μm dia	600 μm dia	200 μm dia	600 μm dia	Thickness of viable rim of 600 μm dia	Histological differentiation	Clinical stage
83	34	27.3	18.3	159	Poorly diff anablastic cer	Ib
75	31	31.0	23.6	168	Mod diff sq ca	IIb
70	21	24.2	17.2	147	Mod diff adeno	Ib
69	26	13.8	11.6	153	Well diff sq ca	IIb
54	9	28.4	3.7	191	Mod diff sq ca	Ib
49	22	24.0	17.4	142	Poorly diff sq ca	Ib
42	6	10.0	9.4	68	Poorly diff sq ca	IIIb
40	16	25.8	14.0	112	Poorly diff sq ca	IIb
36	13	14.0	10.2	102	Mod diff sq ca	Ib
32	11	12.1	10.1	81	Poorly diff sq ca	II
28	5	17.3	6.8	64	Mod diff sq ca	IIb
21	3	9.0	8.3	48	Poorly diff sq ca	I
12	8	11.8	9.2	17	Well diff sq ca	IIb

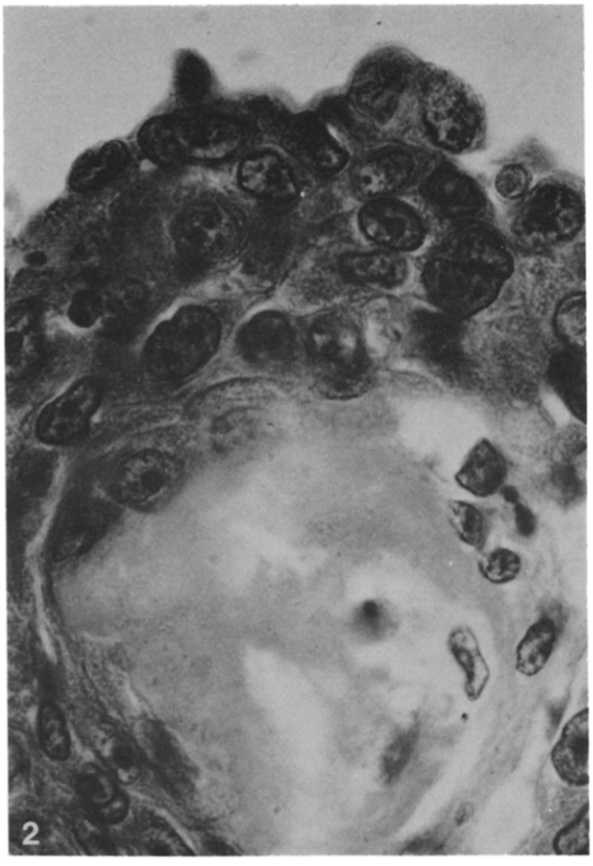
Table 2. Cervical spheroid growth kinetics and response to chemotherapy

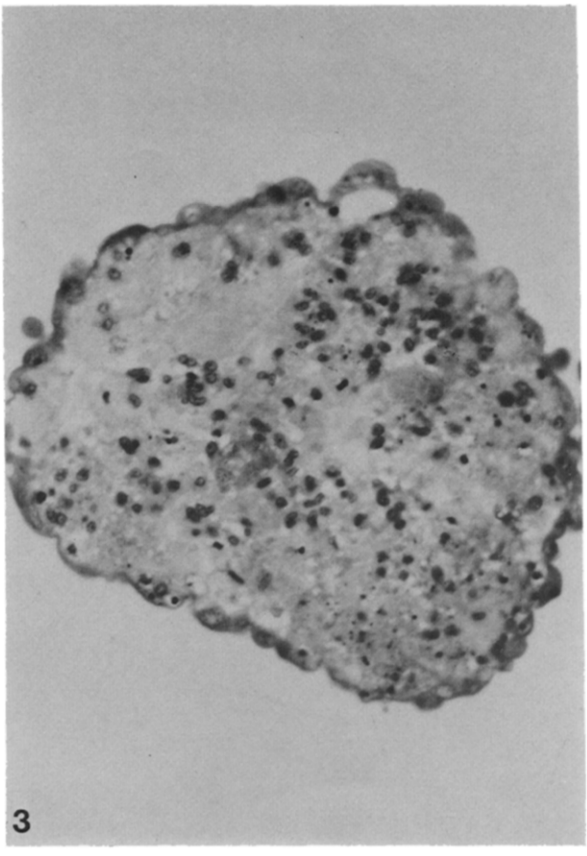
VDT*	LI†	GF‡	TVR§	Act-D	Adr	Ara-C	BCNU	Bleo	Cispl	5-FU	Melphalan	Meth	Mit-C	Vinbl	Vinc	MeCCNU
3.7	20.7		146	–	+	++	–	++	+	+	++	++	–	+	++	–
5.0	13.3		122	–	+	+	+	++	+	++	++	++	–	–	+	+
5.6	18.3		159	–	+	–	++	++	–	–	–	–	–	–	+	–
5.9	23.6	28	168	–	++	–	++	–	+	++	++	++	–	+	–	–
7.8	11.6	23	453	–	–	–	–	–	–	+	–	–	+	–	–	–
8.0	17.4	23	156	–	–	+	++	+	–	++	–	++	–	–	–	+
8.4	19.2	49	130	–	+	–	++	++	–	–	–	–	–	+	+	+
9.3	17.2	32.4	147	–	–	–	–	++	–	+	–	–	–	–	–	–
11.0	14.0		112	–	++	–	–	–	–	++	–	–	–	–	+	–
11.7	16.4	56	162	–	++	–	+	++	–	++	++	+	–	–	–	–
14.2	10.2	9.4	102	–	–	–	–	+	–	–	+	++	–	–	–	–
17.3	10.1	11.1	81	–	–	–	–	++	++	–	–	++	–	–	–	–
20.0	9.2		17	–	–	+	+	–	–	+	–	–	–	–	–	+
24.0	13.6		86	–	–	+	+	+	–	–	+	+	–	–	–	–
25.0	10.0	17	132	–	–	–	–	–	–	–	–	+	–	–	–	–
26.4	3.7		491	–	+	–	–	+	–	–	+	–	–	–	++	–
28.3	9.4		68	–	–	–	–	+	–	++	++	++	+	–	–	–
34.1	8.8	11	92	–	–	–	++	–	–	++	–	–	–	+	+	–
36.0	6.8	18	164	–	–	–	–	–	–	–	–	–	+	–	–	–
43.9	5.1		63	–	++	–	–	++	–	–	–	–	+	–	–	–
51.7	6.8		43	–	–	+	+	++	–	–	–	++	–	–	–	+
54.1	8.3	13	48	–	–	–	+	+	–	++	+	–	–	–	–	–
Not significant				22	13	16	11	7	18	10	13	11	18	18	15	17
Partial response				0	5	5	6	6	3	4	4	3	4	4	5	5
Full response				0	4	1	5	9	1	8	5	8	0	0	2	0

++ 20% Regression)
\+ 10–19% Regression)
– 10% Regression)

*VDT = volume doubling time.
†LI = labelling index.
‡GF = growth fraction.
§TVR = thickness of viable rim (μm).

The significance of these three groups is described under 'Results'. These values represent the maximum observed regression during measurements made following exposure to the cytotoxic drug.





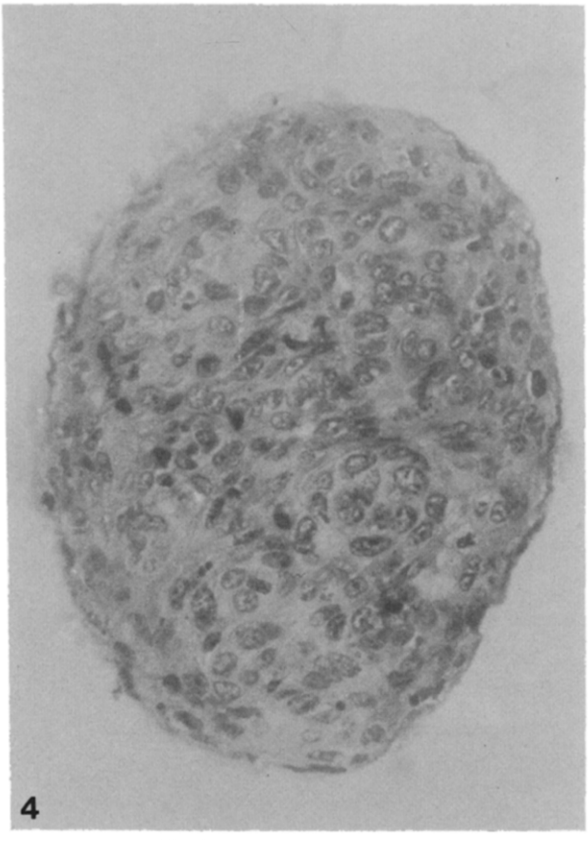


Fig. 2. *Example of viable cell rim and central necrosis in histological section of a spheroid grown from cervical tumour tissue* (*H and E* × 1000).

Fig. 3. *Example of spheroid of cervical tumour cells displaying a single layer of cells at the viable rim* (*H and E* × 320).

Fig. 4. *Example of spheroid of cervical tumour cells without central necrotic core* (*H and E* × 320).

Table 3. Histological grading and clinical staging of cervical tumours and the in vitro *chemosensitivities of the spheroids derived*

	Histology of tumour sample			Clinical staging of tumour		
In vitro response* to	Poorly differentiated	Moderately differentiated	Well differentiated	I	II	III
3 drugs	7	5	1	4	7	2
2 drugs	8	2	0	3	6	1
1 drug	2	7(2)	3	9	3	0
0	0	7(1)	4(1)	2	7	2
Total	17	21(3)	8(1)	18	23	5

**In vitro* response defined as greater than 20% spheroid regression as described in the text. Numbers in brackets denote adenocarcinomas; all others were squamous cell carcinomas.

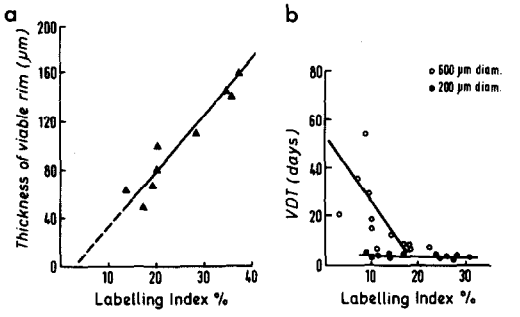


Fig. 5. (a) *Relationship between thickness of viable rim and per cent labelling index (LI). Line drawn by linear regression analysis. Each point represents the mean of three measurements, standard errors of LI did not exceed* ± 6%. (b) *Relationship growth rate (VDT = volume doubling time), and per cent LI. Each point represents mean of three experiments, standard errors of LI did not exceed* ± 3% *for* 200 μm *diameter spheroids, or* ± 8% *for* 600 μm *spheroids.*

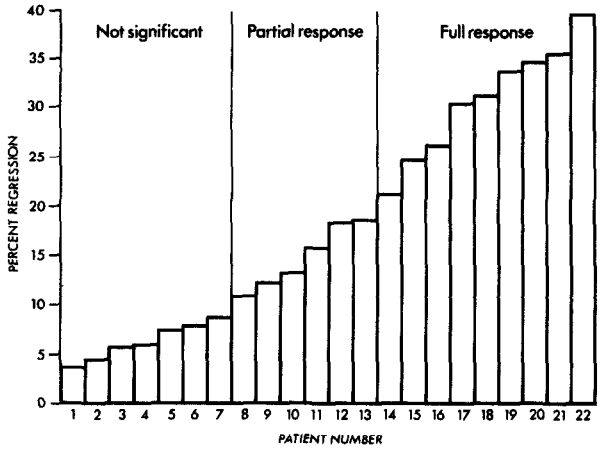


Fig. 6. Response of spheroids, cultured from biopsy tissue from 22 *patients with cervical cancer, to the cytotoxic drug bleomycin. Each value represents the result of a single experiment carried out as described in the text.*

thickness of the viable rim of spheroids, even in the 250–300 μm range, regression as determined by reduction in diameter is not the only criterion that must be applied. Full determination of response requires measurement of mean spheroid diameter of spheroid cultures every 2–3 days following treatment with cytotoxic drugs, for a period of some 30 days. Thus initial regression, if present, will be determined, followed by growth inhibition and regrowth characteristics. Examples of such plots of mean spheroid diameter *vs.* time following drug treatment are shown in Fig. 7. Because of the extent of regression caused by certain of the cytotoxic drugs, larger spheroids (~ 500 μm) were used in this series of experiments.

DISCUSSION

Human cervical cancer cells growing *in vitro* as multicellular spheroids have certain analogies to the microscopic features of those regions of solid tumour *in vivo* where cellular heterogeneity of oxygen status and proliferative state have been demonstrated [15]. The differences in the growth and kinetics of cervical spheroids support the concept that tumours of similar histology may exhibit kinetic heterogeneity [16] and suggest that the growth pattern of cervical spheroids may be influenced by their *in situ* cellular growth capabilities.

Several characteristics of cervical spheroids, e.g. growth rate, labelling index, thickness of viable rim, were similar for drug resistant and sensitive spheroids (Table 2) and therefore appear to be unrelated to *in vitro* chemosensitivity. The histology of the tumour may be more useful with poorly differentiated tumours predicting the better *in vitro* drug responders. However, the data presented do not completely discount some link between cell population kinetics and *in vitro* drug response, since more drugs were active against the faster growing responding spheroids. Since successful chemotherapy depends on a balance between the killing of cells and the proliferation of survivors, it is possible that the true impact of differences in the cellular kinetics of cervical spheroids may have been greater but for the fact that both cell killing

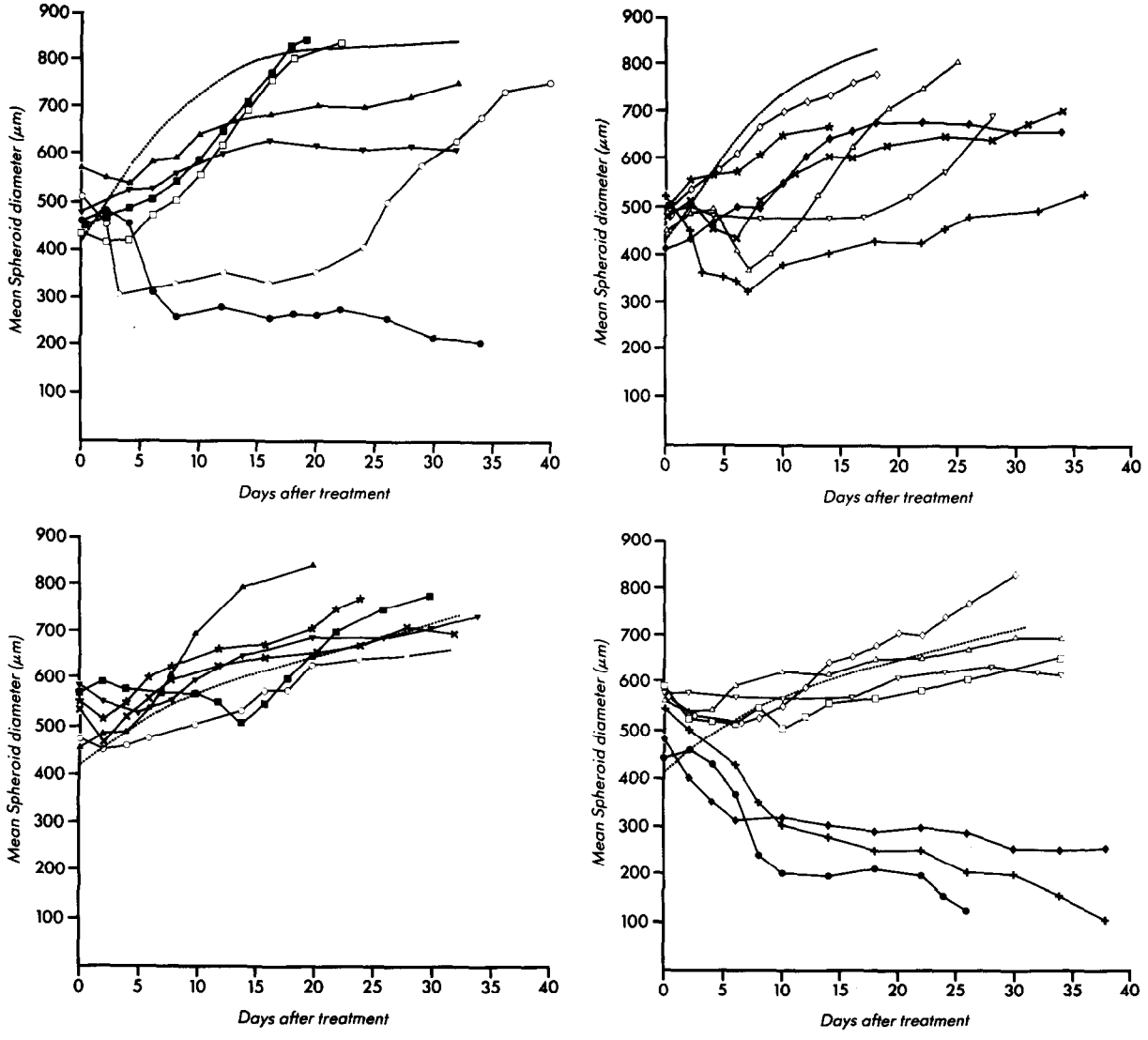


Fig. 7. Response curves showing mean spheroid diameter as a function of time following treatment with a range of cytotoxic drugs. (a) *and* (b) *Response curves for rapidly growing spheroids cultured from a cervical cancer biopsy* (*VDT* = 5 days; *LI* = 18.3%; *GF* = 23.0%; *TVR* = 159 μm). (c) *and*(d) *Response curves for slowly growing spheroids* (*VDT* = 34.1 days; *LI* = 8.8%; *GF* = 11.0%; *TVR* = 92 μm). *Abbreviations specified in Table 2. Symbols* (●) *5-FU*; (○) *BCNU*; (■) *Melph*; (□) *Me-CCNU*; (▲) *Vinb*; (△) *Vinc*; (◆) *Meth*; (◇) *Ara-C*; (▼) *Cispl*; (▽) *Act-D*; (+) *Bleo*; (×) *Adr*; (★) *Mit-C*; (---) *Control*.

and proliferation of survivors are probably rapid in fast growing spheroids. This is supported by the observation (Fig. 5) that cervical spheroids with high proliferative activity have a thicker rim of viable cells which may result in inadequate drug dosage to the inner cells. Our results suggest that another reason the cellular kinetics of cervical spheroids appear to be of limited value in choosing drugs for treatment, is that the inherent sensitivity or insensitivity of the cancer cells may be more important than their rate of proliferation.

The ultimate evaluation of the impact of spheroidal culture on chemosensitivity testing will depend on demonstrating correlation of *in vitro* results with *in vivo* responses in the clinic. Such correlations are now underway with the co-operation of clinical consultants in the Leeds area. Drug penetration experiments are also in progress for correlation with the clinical results. Communication of these data will be made when they reach statistical significance. In particular the action of cisplatin as discussed by Connors [17] would seem a particularly suitable drug to be investigated further by its action on spheroids. However our, admittedly limited, initial results suggest bleomycin, 5-fluorouracil, and methotrexate as more efficatious than *cis*-platinum and a comparison of these agents is envisaged.

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