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Differences in Chemosensitivity Between Subcutaneous and Pulmonary Tumours*

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Abstract—*The response of a murine tumour to cyclophosphamide has been studied in two sites (subcutis and lungs) over a wide range of tumour sizes. The site of tumour growth has been shown to have a marked influence on chemosensitivity for tumours of equivalent size. Pulmonary metastates are much more sensitive than subcutaneous implants. In the lung there is a simple decrease in curability as the tumour grows, presumably reflecting the increase in clonogenic cells per tumour. In the subcutis the pattern is more complex. An initial sensitivity is followed by a decline during the avascular phase of growth. As the vascular network develops the tumours regain their chemosensitivity. There is no correlation between volume doubling time and chemosensitivity. This study indicates that it is impossible to predict the response of pulmonary deposits from a study of subcutaneous implants.*

INTRODUCTION

SMALL rodents are commonly used for comparing the efficacy of different cancer treatments, using serially transplanted tumours growing subcutaneously or intradermally. These implants give rise to tumour nodules which are readily accessible for studying the effectiveness of treatments by means of growth delay or local control at the implant site [1–3]. An implant site can be chosen which will allow tumours to be treated with localised therapy without causing exessive damage to critical normal tissues and organs. Such a model may be appropriate for studies of radiation or hyperthermia, since the clinical application of these modalities will also be localised. However, systemic therapy designed to eradicate disseminated disease, including micrometastases, is also often tested in the laboratory against large subcutaneous tumours. If occult micrometastases respond in the same way as larger localised tumours this is an appropriate model, and some clinical studies are based on this premise [4]. However, several animal experiments have demonstrated that the chemosensitivity of tumours growing in the lung is different from tumours implanted subcutaneously or intramuscularly [5–9]. In these studies size and site have been varied simultaneously and therefore the quesion arises as to whether the difference in chemosensitivity reflects a difference due to site of growth or due to size at treatment. The fraction of cells actively proliferating and the vascular network (necessary for distribution of the drug) will both change with tumour size and may also differ from one site to another [11–13].

We have therefore undertaken a study of a murine tumour which can be grown subcutaneously and which metastasizes naturally to the lung. Tumours have been treated at a range of sizes in both sites in order to study independently the effect of tumour size and site. Some details of the tumour system have been published previously [14].

MATERIALS AND METHODS

The tumour used in these studies (CBA SA F) arose spontaneously at the Gray Laboratory in 1957 [15] and has been maintained since then by serial passage in syngeneic CBA/Ht GyfBSVS mice. Since 1979, i.e. the 650th transplant, cells have been stored in liquid nitrogen, and after each ten transplants fresh cells are taken from the store. It is a rapidly growing anaplastic tumour which can be transplanted subcutaneously with as few as 1–10 cells. This low TD_{50} (the average number of cells per tumour implant to give a 50% take probability) is unaltered if the mice are preimmunized by injecting heavily irradiated

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tumour cells [Begg, unpublished] or by prior growth and excision of a tumour of the same type [Smith, unpublished]. This indicates that there is little or no immunogenicity of the tumour in our CBA mice (i.e. the substrain of origin).

Subcutaneous tumours were grown on the rear dorsum of female mice by injecting a single cell suspension of 10^3 cells. The SA F tumour suspension was made by mincing and filtering the mince from pooled 8-mm diameter subcutaneous tumours. Cells were counted using phase contrast microscopy and cell suspensions were diluted in Hank's Balanced Salt Solution to give 2×10^4 cells/ml. Of this solution, 0.05 ml was injected subcutaneously.

Pulmonary metastases were induced by allowing subcutaneous SA F tumours to grow to 10 ± 1 mm mean diameter before excision. Cells seed spontaneously to the lungs from this site and when the primary tumour was surgically excised the pulmonary tumours could be studied. The subcutaneous tumours were excised under penthrane anaesthesia (methoxyfluorane; Abbot Labs, Queensborough, Kent). The tumour and overlying skin were removed and the wound was closed with surgical clips. Local recurrence was rare (<2%) and the incidence of lung metastases was very high when the tumour was excised at this size (94-100%).

Cyclophosphamide (CY) was obtained in pure form from Ward Blenkinsop Pharmaceuticals, Bracknell, Bucks. It was dissolved in sterile saline (0.9% NaCl) and injected intraperioneally in an appropriate concentration, allowing a constant volume (0.01 ml/g) to be administered at each drug dose. Single doses of cyclophosphamide were administered, ranging from 40 to 230 mg/kg. The maximum tolerated dose is approximately 230 mg/kg for this strain of mice [16]. Animals with subcutaneous tumours were treated at 2, 4, 6, 8 and 10 days after implantation. Animals with pulmonary metastases were injected with CY immediately after excision of the primary tumour, or 3, 6, 9 or 12 days later.

Subcutaneous tumours

The response was assessed by measuring regrowth delay and local control. All tumours were measured 2-3 times a week across three orthogonal diamaters. The geometric mean diameter was calculated for each tumour and the time taken to reach a mean of 6 mm was used as a measure of response. This time was averaged for all the tumours in each dose group (7-10 mice). The delay induced by CY treatment was calculated by subtracting the time taken for control tumours to reach 6 mm. Animals with no evidence of tumour 70 days after treatment were presumed to be curved. Dose-response curves could be constructed for growth delay or for local control.

Pulmonary tumours

After excision of their primary tumours mice with lung metastases were checked daily for signs of respiratory distress, an indicator of excessive pulmonary tumour burden. Untreated mice were killed at preset intervals (3-15 days after excising the primary) and the number and size of superficial lung metastases in each mouse was determined as follows: the lungs were removed, fixed in Bouin's solution and visible metastases were counted and measured using a binocular dissecting microscope (magnification × 6).

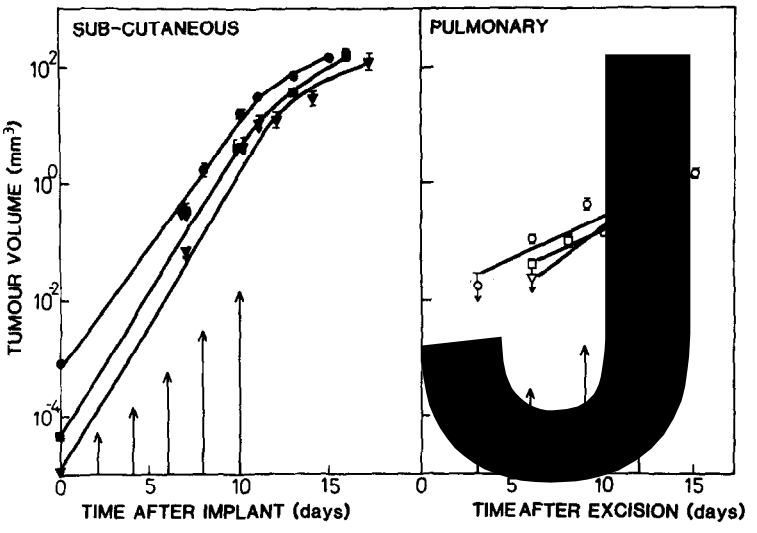

Fig. 1. Growth curves for subcutaneous and pulmonary tumours. Three separate experiments are shown. Each point represents mean ± 1 S.E.M. of 8-10 animals (subcutis) or 30-170 tumours (pulmonary). Growth in the lung is slower than that in the subcutaneous site over the size range 0.1-1.0 mm³. Tumours were selected for treatment at the times indicated by the arrows.

Tumour volumes were calculated assuming the tumours to be spherical. Mice were killed at the first sign of respiratory distress. If the treatment was sufficiently effective to eradicate all pulmonary and other tumours the mice showed no distress and were killed at a fixed time (120 days) after CY administration. Dose-response curves for CY treatment could be constructed for three endpoints: (a) % of animals cured of all tumour at 120 days; (b) number of metastases/mouse at termination; and (c) % of individual metastases locally controlled (by comparing the average number of tumours in the lungs of untreated mice with the average number in treated mice at termination).

RESULTS

Figure 1 shows the growth curves for subcutaneous and pulmonary tumours from three separate experiments. The subcutaneous tumours are plotted against time from implantation. The three sets of data are displaced in proportion to the number of cells required to give 63% takes in three experiments. The point at zero time represents the initiating volume of the clonogens in the implanted mass, obtained from the TD_{63}, i.e. the average number of cells per tumour implant to give rise to a 63% take probability (80, 21 and 1 cell respectively for experiments A, B and C), and the average volume of each tumour cell (12 μm diameter, 905 μm^3 volume, estimated with a Royco cell counter). Smooth curves have been drawn by eye through the measured data and these 'initiating volumes'. The volumes at treatment are indicated by arrows, and are listed in Table 1. The volume doubling times have been derived from the tangent to the curve at each size, and show slower growth as the tumours enlarge.

The growth of pulmonary metastases is shown in Fig. 1b plotted against the time at which the mice were killed relative to the excision of the primary tumour. Each point results from the mean of the volumes of 30–170 lung tumours. At 3 days (and in one experiment at 6 days) after excision there were fewer metastases per mouse than at the later termination times. Thus some of the seeded tumours were probably too small to be detected and the mean volumes must be biased towards high values; these points are indicated by the downward arrows. The growth rates are consistently slower in the lungs than in the subcutis for a comparable-sized tumour.

In experiment A an average of 16.4 tumours eventually developed per mouse (range 6–34); in experiment B the average number of metastases per mouse was 14.4 (range 0–31). In both experiments these values remained constant from 6 to 14 days, indicating that all seeded tumours

Table 1. Characteristics of tumours at treatment

Site	Treatment time (days)	Treatment size (volume, mm^3)	Estimated cell number*	Volume doubling time (days)
Subcutaneous implants	(from implant to CY injection)			
	2	0.0001[a]†	111	0.6
	4	0.0012[a]†	1326	0.6
	6	0.014[a]†	15,000	0.6
		0.07[b]†	77,000	0.65
		0.26[c]†	290,000	0.75
	8	0.45[b]	500,000	0.65
		1.75[c]	1,900,000	0.7
	10	1.8[a]	2,000,000	0.7
		4.4[b]	4,900,000	0.8
		12.0[c]	13,000,000	0.85
Pulmonary deposits	(from excision of 10 mm primary)			
	0	<0.0005[a]	<579	—
	3	<0.02[a]	<22,000	<1.3
	6	0.02[b]	22,000	<1.2
		0.04[c]	44,000	1.7
		0.11[a]	120,000	1.45
	9	0.45[a]	500,000	—
	12	0.39[a]	430,000	2.0
		0.6[b]	660,000	—

*Estimated from volume, assuming cells are 12 μm diameter, no necrosis.

†Obtained by back extrapolation of curves. Data are shown from three separate experiments: a = first experiment; b = second; c = third.

were visible by 6 days. No plateau in the number of metastases was seen, however, in a third experiment (C) for mice killed at 6-12 days after primary exicision. The estimated sizes and the volume doubling times for the tumours from all three experiments at treatment are listed in Table 1.

Subcutaneous tumours

Figure 2 shows examples of the response of subcutaneous tumours treated with single doses of 80 or 160 mg/kg CY at different sizes. The rates of regrowth of treated tumours were similar to controls for all CY doses and the size of the tumours at treatment appeared to have little or no influence on the overall growth delay induced by the drug.

Figure 3 shows the regrowth delay data for tumours treated at 6, 8 and 10 days with graded doses of CY. Data are not shown for the earlier times of 2 and 4 days because many tumours were cured at all CY doses. The delay in growth to 6 mm diameter is shown in each panel, and is approximately linear with dose. The hatched line in panels B and C is reproduced from panel A to show that the growth delay is similar at the three treatment sizes. Data from three separate experiments are included. Upward arrows are shown when the indicated fraction of mice had their tumours locally controlled; these were allocated an arbitrary growth delay of 55 days, corresponding to the latest time at which a tumour was seen to regrow after CY treatment [17].

Pulmonary tumours

Figure 4 shows the dose-response curves for control of lung metastases in animals treated with CY at different times after surgical excision of the primary tumour. The estimated cell number in each set of tumours at the time of treatment is indicated against each line. The percentage of cured mice increased with increasing CY dose at all treatment sizes. However, animals were more readily cured when treatment was given soon after

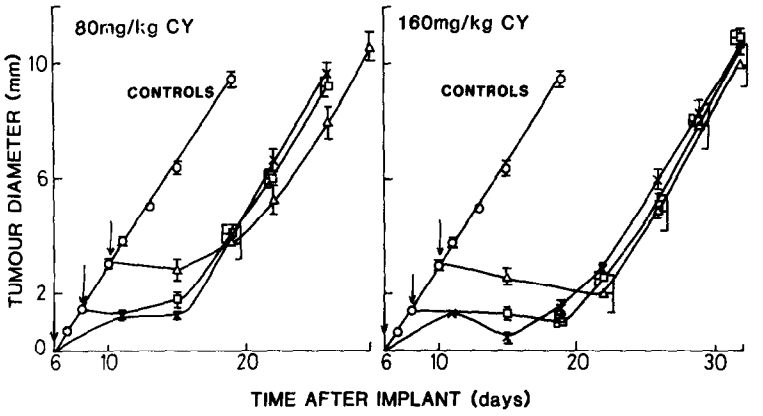

Fig. 2. Growth curves for subcutaneous tumours, untreated or after receiving 80 or 160 mg/kg cyclophosphamide at the sizes indicated by the arrows. The response to each drug dose was similar for the three treatment sizes. Each point represents mean ± 1 S.E.M. for 8-10 mice.

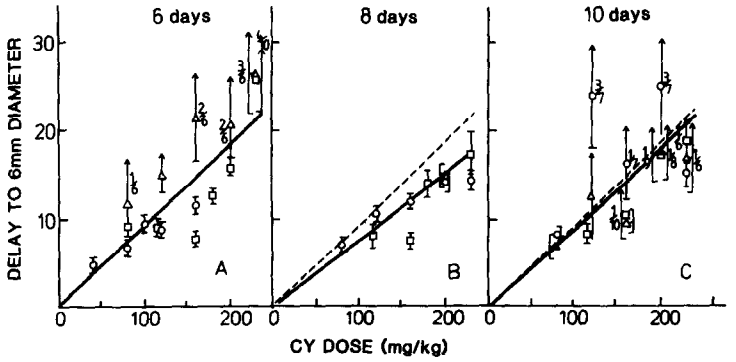

Fig. 3. Growth delay (relative to untreated controls) as a function of cyclophosphamide dose. Subcutaneous tumours treated 6, 8 or 10 days after implantation. The delay induced by CY is approximately proportional to dose and is similar at the three treatment times. The line from panel A is reproduced (dashed line) in panels B and C for comparison. Each point represents the mean of 6-10 mice ± S.E.M. Upward arrows show groups in which the indicated number of tumours were locally controlled (see text).

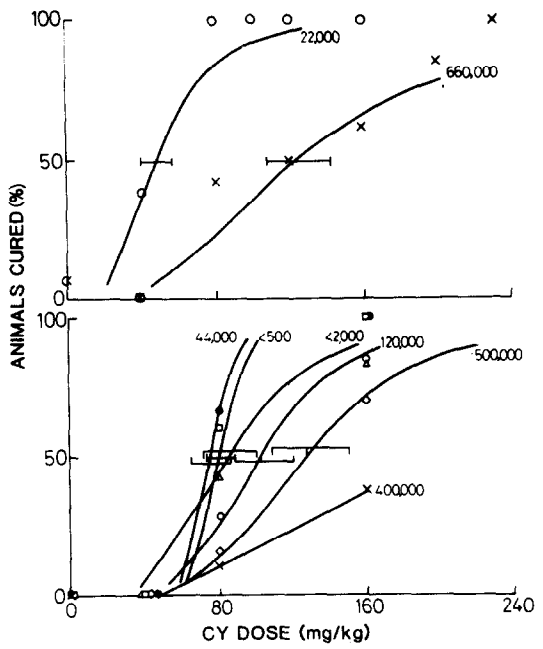

Fig. 4. The response of pulmonary metastases to graded doses of cyclophosphamide assessed as cure of all metastases in each mouse. The tumours become more difficult to cure as they increase in size. (The estimated cell numbers are indicated against each curve.) The lines are fitted by logit analysis and the TCD_{50} *(cyclophosphamide dose to give 50% long-term animal survival) ± 1 S.E.M. are shown. Upper panel: experiment B, in which less than 100% of the untreated mice developed metastases; lower panel: experiments A and C combined.*

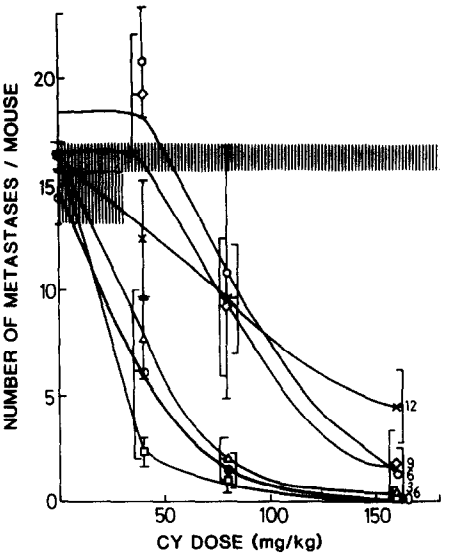

Fig 5. Control of individual pulmonary metastases as a function of cyclophosphamide dose. The number of metastases per mouse (at termination) decreased with increasing dose. Tumours treated 0, 3 and 6 days after excising the primary tumour were much more sensitive than those treated at later times. Each point represents mean ± 1 S.E.M. of 3-9 mice. Hatched areas represent 1 S.E.M. range for untreated mice in each experiment.

surgery, i.e. when the pulmonary tumour burden was smallest.

Mice killed with lung metastases were frequently found to have other tumours within the thoracic cavity. The origin of these tumours is unknown; it is not clear whether they are secondaries seeded from the primary tumour via lymph channels or tertiary metastases from the lung secondaries. They seemed to show a similar sensitivity to CY as those within the lung and consequently did not obscure the response of the lung metastases when assessed from the patterns of survival in treated animals.

Figure 5 shows the number of metastases observed in treated mice that were killed at 120 days or at earlier times because of respiratory distress. In untreated mice averages of 16.4 and 14.4 metastases per mouse were recorded at termination. The data show a decrease in the incidence of pulmonary metastases with increased CY dose for all the groups. However, tumours treated at earlier times, i.e. when they were smaller, were more sensitive than those treated at later times.

The third analysis of the metastasis data, i.e. the percentage of individual pulmonary tumours which were controlled, is shown in Fig. 6A. This was derived from the number of animals cured (Fig. 4) and the average number of tumours per mouse for each dose level (Fig. 5). For tumours containing 100,000 cells or more (i.e. >0.1 mm^3) there was no significant separation of the curves, although there was a trend towards flatter curves, suggesting a greater variability, in the response of tumours treated at the later times. The subcutaneous tumour data are shown for comparison in panel B. These tumours were much more resistant that the lung metastases when assessed in terms of the dose needed to control 50% of the tumours.

DISCUSSION

Previous publications [6-9] have indicated that tumours growing as small lung metastases may respond better to chemotherapy than larger primary tumours grown in more superficial sites. However, in each of these previous studies the tumours differed both in the site of growth and in the tumour volume at treatment. It has been shown that, after the initial growth of blood vessels, the relative vascularity of a tumour decreases with increasing tumour volume [12, 18] and that growth fraction also decreases with increasing size [10, 11]. It was therefore difficult to determine whether the observed differences in chemosensitivity of tumours in the two sites are due primarily to changes in cell kinetics or in drug delivery, both of which are influenced by the vascular network. In the present studies we have compared the response of lung metastases with subcutaneous tumours of similar size. In general,

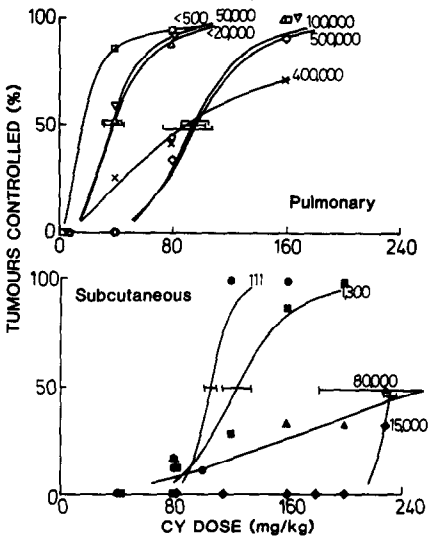

Fig. 6. Local tumour control for pulmonary metastases (upper panel) and subcutaneous tumours (lower panel) as a function of CY dose. (Data derived from the product of tumours per mouse and percentage of mice cured for lung metastases.) For equivalent numbers of tumour cells (as indicated against lines) the lung metastases were much more chemosensitive than the subcutaneous tumours. Logit fitted lines with $TCD_{50} \pm 1$ *S.E.M. are shown.*

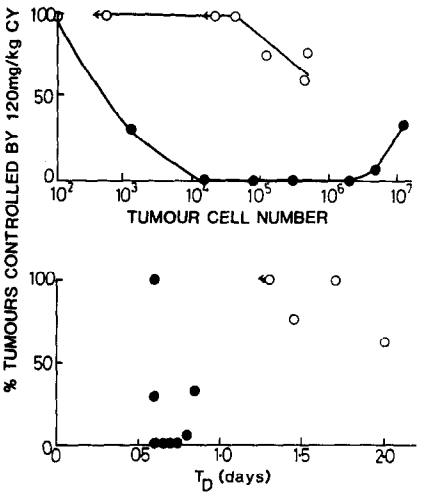

Fig. 7. Comparison of the response of subcuntaenous (●) and pulmonary (○) tumours to a fixed dose (120 mg/kg) of cyclophosphamide. Upper panel: tumour control as a function of estimated number of cells per tumour. Pulmonary tumours are more sensitive at all sizes. Subcutaneous tumours show a biphasic response in chemosensitivity; lower panel: tumour control is poorly correlated with volume doubling time.

in both sites the smallest tumours were most responsive to treatment, presumably reflecting the number of clonogens which must be killed to attain cure. A direct comparison of the sensitivity in the two sites is shown in Fig. 7A. The proportion of tumours controlled by a fixed dose of 120 mg/kg CY is plotted as a function of the estimated number of tumour cells at treatment. There is a clear difference in sensitivity in the two sites if tumours of the same size are compared. Some local control of lung metastases was achieved at all treatment sizes and many mice bearing multiple metastases were cured of their entire tumour burden. By comparison, single subcutaneous tumours treated at similar volumes were seldom controlled by cyclophosphamide. The lung metastases in Fig. 7 show a simple decline in curability as the number of cells present in the tumour increases. The response of the subcutaneous tumours is, however, more complex. At small tumour sizes (i.e. <0.001 mm^3) tumours can be cured with 120 mg/kg CY. At intermediate sizes (0.07–1.8 mm^3) they become incurable, but show a subsequent increase in sensitivity when treated at larger sizes (4–12 mm^3). This increase in sensitivity occurs above the maximum size range available as lung metastases and its parallel cannot therefore be studied in the pulmonary tumours. It is difficult to ascertain what this biphasic pattern of sensitivity is due to since the tumours treated at 2–6 days after implantation are too small to determine drug concentrations, cell kinetics parameters or vascular volumes. However, we have studied tumours in the size range corresponding to the second increase in sensitivity [Begg *et al.*, in preparation]. ^{51}Cr-labelled red blood cells have been used to determine the vascular volume, using the technique described by Song and Lewitt [19]. No vascular space is detectable in tumours less than 4 mm^3 (i.e. containing 10^6 cells) and above that size it increases approximately linearly. This phase of increasing vascular volume correlates with the cell number at which an increase in chemosensitivity is seen in Fig. 7. It therefore seems likely that the initial decline in sensitivity reflects the growth of the tumour in its avascular phase, during which time drug access will be poor. Corresponding vascular studies are not available for the pulmonary tumours, because of their small size.

The lung tumours used in these studies were a true metastatic population resulting from natural dissemination of cells from an implanted subcutaneous primary. As with any other naturally metastasizing system, this has the disadvantage that the actual time of establishment of each individual lung tumour is unknown. However, the system avoids the danger that tumours growing in the lung following intravenous injection of a large bolus of cells may differ in characteristics from tumours which are the end result of the natural metastatic process [14, 20, 21].

At any given size the doubling times of the pulmonary tumours were approximately twice as

long as those grown subcutaneously (Table 1, Figure 1). The data in Fig. 1B have been fitted with straight lines as a first approximation, though it seems more likely that growth will be Gompertzian, as demonstrated in some studies [11, 6]. Exponential growth has been reported for lung metastases up to 50 mm^3 [22], but in the present case back-extrapolation of the lines in Fig. 1B would suggest that the tumours were seeded before the primary tumours reached 4 mm in diameter, and it is known that excision at this size leads to no lung metastases. This slower growth in lungs is surprising since the lung is well vascularised, well oxygenated and offers less mechanical constriction than the subcutis. However, Alexander and Eccles [23] have recently postulated that the oxygen tension in the lung may be too high to favour tumour growth, as has been demonstrated by Joyce and Vincent [24] and Courtenay and Mills [25] for human tumour cells growing *in vitro*. Many malignant cell lines grow better in cultures equilibrated with 5% oxygen than when they are equilibrated with air.

The reason for the greater sensitivity of pulmonary metastases, in spite of their slower growth, is not understood. Some studies have shown that proliferating cells are twice as sensitive to CY as cells in plateau phase [26, 27], although others have shown no proliferation dependence [28]. In contrast to the present results, de Wys [7] has shown little variation in sensitivity with site of implant but a big decrease in sensitivity with size, which he correlated with growth rate. Figure 7b shows that our data do not conform to that pattern.

The response of any tumour to a chemotherapeutic agent is a complex function of number of clonogens, drug access, pharmacokinetics in the tumour micro-environment and proliferative status of the cells. This study has shown that the balance between these factors changes in a different way in the two sites as the tumour enlarges. At equivalent sizes the tumour sensitivity differs markedly at the two sites. It therefore seems unwise to assume that the response of a tumour in a readily accessible site such as the subcutis can be used as an indicator of the sensitivity of metastases in different sites elsewhere in the body.

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