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Hyperthermia Response and Thermotolerance Capacity of an Experimental Rat Tumour with Occluded Blood Flow

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Abstract—*Thermotolerance* in vivo *was investigated using the rat fibrosarcoma SSBla. Hyperthermia was administered using water-bath heating, with the tumour blood supply occluded by clamping during the heating procedure. Single-dose heat treatments* (10–60 min *at* 43.5°C) *yielded linear dose–response curves, with response evaluated as tumour re-growth delay. In thermotolerance studies split-dose treatments were given in which a single 'priming' treatment of* 30 min *at* 43.5°C *was followed* 24 *or* 48 hr *later by a variable treatment* (10–90 min), *again at* 43.5°C. *Split-dose responses produced generally less uniform results, though the dose–response curves were still approximately linear. Comparison of the slopes of the dose–response curves for single-dose and split-dose treatments gave a 'thermotolerance ratio' (TTR) of* 4.09 *and* 3.45 *at* 24 *and* 48-*hr intervals respectively. Data analysis using Monte Carlo simulation techniques also suggests that the distribution of tumour sensitivities to a second heat treatment may be considerably broader than the distribution of sensitivities to a first treatment. Uniformity of tumour response to a single heat treatment may therefore conceal a spectrum of capacities for development of thermotolerance amongst individual tumours.*

INTRODUCTION

IN CONSIDERING the use of hyperthermia in the treatment of cancer it is necessary to evaluate the response of tumours and of relevant normal tissues to both single- and multiple-heat treatments. The response to fractionated hyperthermia is, however, influenced by the phenomenon of 'thermotolerance'—a transient resistance to heat which may be induced by a previous heat treatment. Thermotolerance is well-documented for cells in culture and for some normal tissues [1, 2], but relatively few studies of tumours *in vivo* have been reported. The effect of hypoxia (or hypoxia-associated conditions, e.g. low pH) on the development of thermotolerance is also uncertain, and has been reported either to diminish [3] or to enhance [4] thermotolerance.

In this paper we report the hyperthermia response and development of thermotolerance in the rat fibrosarcoma SSBla, with tumour response evaluated as re-growth delay. Throughout treatment the tumour blood supply was occluded by mechanical clamping, eliminating tumour cooling by blood flow but rendering the tumour cells hypoxic and perhaps depriving them of nutrition. By comparing the mean slopes of the growth delay versus heating time curves for single- and split-dose treatments we have estimated a mean 'thermotolerance ratio' (TTR) for intervals of 24 or 48 hr between treatments. Using Monte Carlo simulation techniques, we have also estimated the relative widths of the distribution of heat

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sensitivity shown by these tumours to first or second treatment.

MATERIALS AND METHODS

The tumour used was the fibrosarcoma SSBla, whose biological properties have been described previously [5]. Briefly, the tumour originated spontaneously in the mammary tissue of a female Johns Wistar rat but was subsequently propagated serially in Wistar/CFHB hybrid rats of both sexes. The tumours were surgically implanted (as 1-mm cubes) subcutaneously in the mid-dorsal region and the incision was closed with a surgical clip which was removed 3 days later. The transplantation 'take rate' was 95%. Following a latent period of 7–14 days, tumour growth was monitored three times weekly by measurement of three mutually perpendicular dimensions using calipers. Tumour growth was exponential in form (volume doubling time ≈ 2 days) in the size range of tumour mean diameters ranging from 5–20 mm, with Gompertz retardation becoming evident thereafter. Distant metastases were occasionally found in animals 'locally cured' by radiation or hyperthermia, but with very low frequency. The tumour is fibrous in composition and not easy to disaggregate, which prevents the use of the TD_{50} assay to quantify immogenicity. 'Crude' tests for immunogenicity based on 'take rate', latency and growth rate of tumours implanted (as 1-mm cubes) into animals 'cured' of previous tumours by X-irradiation of hyperthermia showed no differences from controls. Spontaneous regressions were not seen. It therefore seems unlikely that the SSBla fibrosarcoma is 'strongly' immunogenic in these host animals, but 'weak' immunogenicity cannot be excluded.

Animals were assigned to treatment groups when the geometric mean diameter of the tumour was in the range 7–10 mm. In all experiments male and female rats were used in roughly equal proportions.

Hyperthermia treatment procedures were as described previously [6]. Animals were anaesthetised by i.p. injection of neuroleptanalgesic mixture ('Hypnorm',, Crown Chemical Co. Ltd.) at 1 mg/kg for males, reduced by 8% for females [7]. Anaesthetised animals were placed supine on a plastic tray in which slots had been cut, and the tumours and surrounding tissue allowed to protrude through the slot. The protruding tumours were then held in plastic clamps designed to occlude blood flow and the clamps tightened with Perspex screws. Typically, no more than 5 min elapsed between the tightening of the first and last screws at the beginning of treatment, the removal of clamps at the end of treatment following a similar time-scale. Six rats could be accommodated on one such tray, which was then placed over a precision-controlled water bath, pre-set to 43.5°C, with the tray supported on the sides of the bath, the clamped tumours submerged and the bodies of the rats above the surface of the water. In a separate study, thermocouple probes were inserted into the tumour and found to stabilise at 0.2–0.5°C below water-bath temperature within 2–4 min.

Detailed studies of intra-tumour temperature distributions were not, however, attempted. During treatment, animal rectal temperature typically rose to 39–40°C, falling, however, to sub-normal temperatures (30–33°C) after cessation of treatment. In a minority of animals rectal temperatures remained low 24 hr later and a very small proportion of animals ($\approx 2\%$) died at this time, possibly from this cause.

After treatment for a fixed time the rats were removed, dried and allowed to recover. For single-treatment groups various heating times (10–60 min) were employed. For split-treatment groups the first 'priming' treatment was always of 30 min duration. Then, 24 or 48 hr later, the procedure was repeated and the rats subjected to a second treatment at 43.5°C for 10–90 min.

After treatment tumour size was regularly monitored and re-growth curves constructed for each individual tumour. For hyperthermia of SSBla, the mean slope of the growth curves for untreated tumours is identical to that of the curve for heated tumours [6]. Growth curves were recorded for each individual recurrent tumour and quantified by fitting a straight line (method of least squares) relating the logarithm of geometric mean diameter to time since treatment. Re-growth delay was calculated for each individual tumour as time to reach 15 mm geometric mean diameter, and the mean re-growth delay calculated as the arithmetic average of the individual re-growth delay times. This 'individualised' procedure was followed in order to avoid artefacts associated with construction of volume-averaged 'composite' growth curves, which have been shown [8] to be biased toward slow-growing tumours due to earlier (humane) sacrifice of animals bearing fast-growing tumours.

For 'sham hyperthermia', animals were anaesthetised and their tumours clamped for 60 min, but without exposing them to hyperthermia. The mean time to 15 mm for these tumours (5.9 days) was not significantly different from that of control tumours (6.2

days), indicating no effect of clamping only on tumour growth.

In the treated groups few tumours recurred more than 30 days after treatment and in no case more than 42 days later. Non-recurrence by 60 days was adopted as the criterion for 'tumour cure'. Such cures occurred sporadically within the treatment groups, but animal numbers were not sufficient for TCD_{50} analysis. In the present study tumour cures were accommodated in the growth delays by the 'long delay' procedure [9, 10] by which a time close to the upper limit of observed recurrence times (here 30 days) is assigned to non-recurrent tumours. This procedure has been shown [11] to provide satisfactory corrections for homogeneous tumour systems, though the correction becomes less accurate in the presence of heterogeneity.

RESULTS

The results obtained are summarised in Table 1. As may be seen, the cure-corrected growth delay results and the uncorrected growth delay results (i.e. excluding cures) show similar trends in dose–response. However, the corrected data have been adopted as probably more representative of average tumour response [9–11]. The cure-corrected dose–response relationships are presented graphically for single-dose treatments in Fig. 1, for split-dose treatments with 24-hr intervals in Fig. 2 and for split-dose treatments with 48-hr intervals in Fig. 3.

As may be seen, the single-dose results show an almost linear response with dose. The 48-hr interval split-dose results show rather more scatter and the 24-hr interval split-dose results show considerably more scatter, but in each case the dose–response pattern is approximately linear. In all three cases best straight lines were fitted to the data by the method of least squares; these best lines are shown in the diagrams. The slope of such a line provides a measure of tumour cell sensitivity (an effective 'D_0' value) and the ratio of split-dose to single-dose slopes provides a 'thermotolerance ratio' (TTR). The individual slopes and derived TTR values are presented in Table 2. For split-dose groups (24- and 48-hr intervals), TTR values of 4.09 ± 0.05 and 3.45 ± 0.03 respectively are obtained.

In addition to the reduced slopes of the split-dose response lines, however, it is noticeable (especially for the 24-hr interval data) that the results are more scattered for split than single treatments. Since the number of animals per dose point is large (19–52), it is improbable that this increased scatter results from small sample effects or from variability of growth rate. We have therefore considered the possibility that the distribution of heat sensitivities to a second treatment is broader than the distribution of sensitivities to a first treatment (e.g. due to variable development of thermotolerance in individual tumours).

In order to quantify the different widths of the sensitivity distributions implied by this hypothesis, we have made use of Monte Carlo simulation techniques (see Appendix). Briefly, the correlation coefficient of the best-fitting line to the dose–response data provides a measure

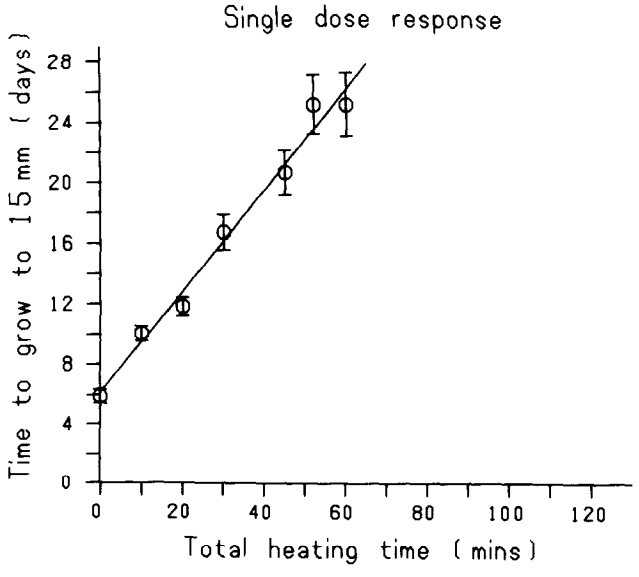

Fig. 1. Relation of cure-corrected re-growth delay (time to grow to 15 mm geometric mean diameter) to treatment time at 43.5°C water-bath temperature. The error bars are standard errors of the mean.

Table 1. *Mean re-growth delay and proportion cured for the rat sarcoma SSBla subjected to hyperthermia at* 43.5°C

Total treatment time (min)	Single treatment				Split treatment, 24-hr interval				Split treatment, 48-hr interval			
	No. of animals	Proportion cured	Re-growth delay (days)	Cure-corrected delay (days)	No. of animals	Proportion cured	Re-growth delay (days)	Cure-corrected delay (days)	No. of animals	Proportion cured	Re-growth delay (days)	Cure-corrected delay (days)
0	21	0	5.9	5.9	—	—	—	—	—	—	—	—
10	22	0.05	9.0	10.1	—	—	—	—	—	—	—	—
20	19	0.105	9.7	11.9	—	—	—	—	—	—	—	—
30	23	0.174	13.9	16.7	23	0.174	13.9	16.7	23	0.174	13.9	16.7
40	—	—	—	—	15	0	15.1	15.1	9	0.44	9.5	18.5
45	27	0.185	18.5	20.7	—	—	—	—	—	—	—	—
50	—	—	—	—	18	0.167	15.2	17.7	11	0.363	14.6	19.7
52	52	0.44	21.4	25.2	—	—	—	—	—	—	—	—
60	48	0.33	22.7	25.2	19	0.315	20.3	23.3	21	0.05	16.0	16.7
70	—	—	—	—	18	0.278	21.6	23.9	20	0.15	16.1	17.5
80	—	—	—	—	22	0.182	21.0	22.6	19	0.195	18.7	20.9
90	—	—	—	—	25	0.20	21.0	22.8	20	0.30	20.8	23.1
120	—	—	—	—	20	0.20	20.2	22.2	17	0.42	23.8	26.0

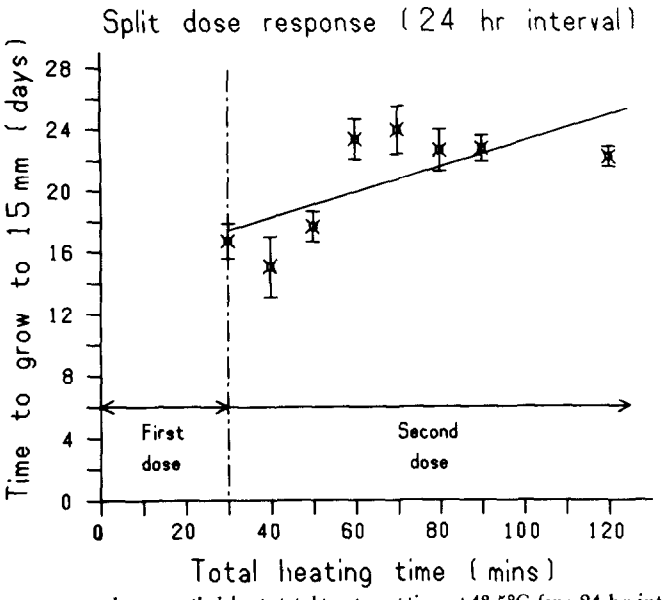

Fig. 2. Relation of cure-corrected re-growth delay to total treatment time at 43.5°C *for a* 24-hr *interval between the first* (30 min) *and the second (variable) treatment. The error bars are standard errors of the mean.*

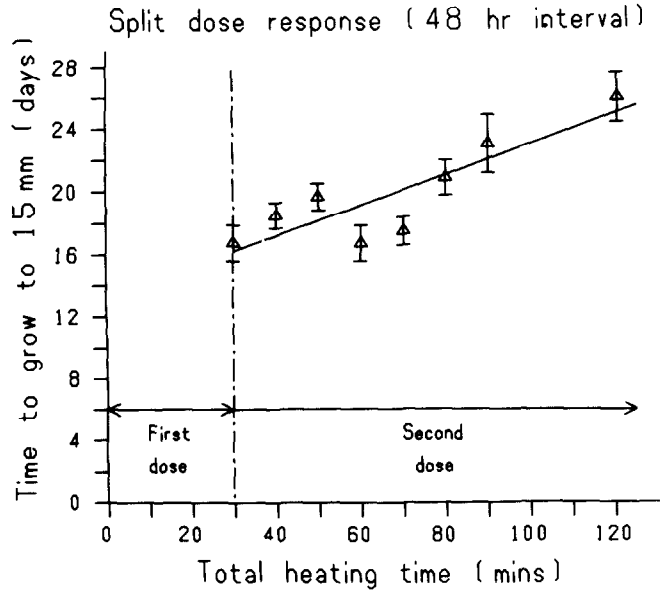

Fig. 3. Relation of cure-corrected re-growth delay to total treatment time at 43.5°C *for a* 48-hr *interval between the first* (30 min) *and the second (variable) treatment. The error bars are standard errors of the mean.*

of the variance of the underlying sensitivity distribution, here assumed normal in form. Figure 4 shows the resultant sensitivity distributions when the data are analysed in this way. Evidently, the variances of the sensitivity distributions are greater for the split-dose experiments, implying, perhaps, that the surviving cells in different tumours have acquired thermotolerance to a variable degree following a priming treatment which achieved a similar level of cell kill in each of the tumours.

DISCUSSION

In employing the growth delay end-point of tumour response it is necessary to be aware of its limitations. Tumour cells constitute only one element of measured volume of a tumour and the clonogenic cells may be only a small fraction of the total. In regarding an end-point such as growth delay as representative of the response to treatment of the clonogenic tumour cells it is necessary to assume that all tumours of the same size contain the same proportion of clonogenic cells, an assumption which, in the case of hyperthermia, could be nullified by a significant contribution by post-treatment oedema to the measured tumour size. However, in the present experiments

Table 2. Linear regression analysis of dose–response relationship for single-treatment and split-treatment hyperthermia

	Single treatment	Split treatment, 24-hr interval	Split treatment, 48-hr interval
Slope	0.326 ± 0.007/day	0.082 ± 0.012/day	0.097 ± 0.008/day
Correlation coefficient	0.992	0.697	0.922
Thermotolerance ratio (TTR)*	—	4.09 ± 0.05	3.45 ± 0.03

*$\text{TTR} = \frac{\text{slope of split-treatment dose–response curve}}{\text{slope of single-treatment dose–response curve}}$.

All stated errors are standard errors of the mean.

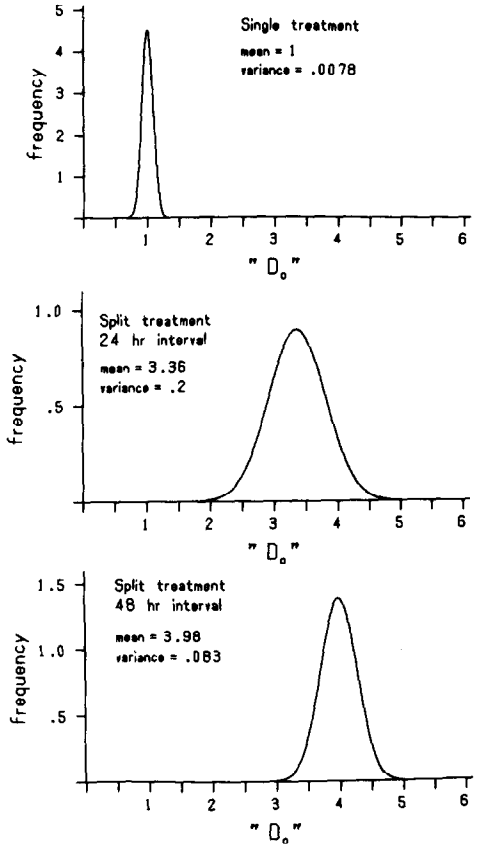

Fig. 4. Distributions of tumour sensitivity ('D_0') to heat, deduced from quality of linear dose–response relationships for each of the three experimental groups. The 'D_0' units are arbitrary and result from taking the mean 'D_0' for the single-treatment group to be unity.

severe oedema occurred only in the higher dose groups and had usually resolved by the time the tumour had reached the end-point size—which was 6–8 times the treatment size, making a significant contribution by oedema seem unlikely. The 'tumour bed effect', a factor which complicates the analysis of tumour response to ionising radiation, does not seem to occur for hyperthermia, the growth rates of tumour recurrent after hyperthermia not being different from those of control tumours [6]. It seems reasonable to suppose, therefore, that the growth delay end-point is no less reliable in the case of hyperthermia than of other modalities, though studies of the relation of the growth delay response to clonogenic cell survival (where measurable) would seem to be indicated. Here we suppose that the growth delay response is (at least approximately) representative of clonogenic survival and that this may be used to quantify the cell-killing effect of hyperthermia and the development of thermotolerance.

Though a considerable body of data now exists on thermotolerance of cells in culture and of some normal tissues *in vivo* [1, 2], information on the development of thermotolerance in tumours has only recently begun to accumulate [12–14]. Despite its obvious importance, direct comparison of the magnitude of thermotolerance in normal and malignant tissues is by no means straightforward.

For cells in culture, thermotolerance has been defined as an increase in the D_0 of the survival curve with or without an increase in the shoulder [1], though this definition has been criticised as unduly restrictive [2]. For tumours, reported studies have utilised either the TCD_{50} ratio for single- and split-dose treatments or the ratio of slopes of the growth delay dose curve as tumour response end-point. For tumours which conform to Poisson cure statistics and whose re-growth curve is unaltered by treatment, each of these corresponds to a D_0 ratio [15]. For normal tissues, however, a commonly employed end-point is the ED_{50} ratio (i.e. the ratio of doses causing the same degree of damage, usually assessed on an arbitrary scale, in 50% of animals). It is a feature of the ED_{50} assay that it often depends on the level of

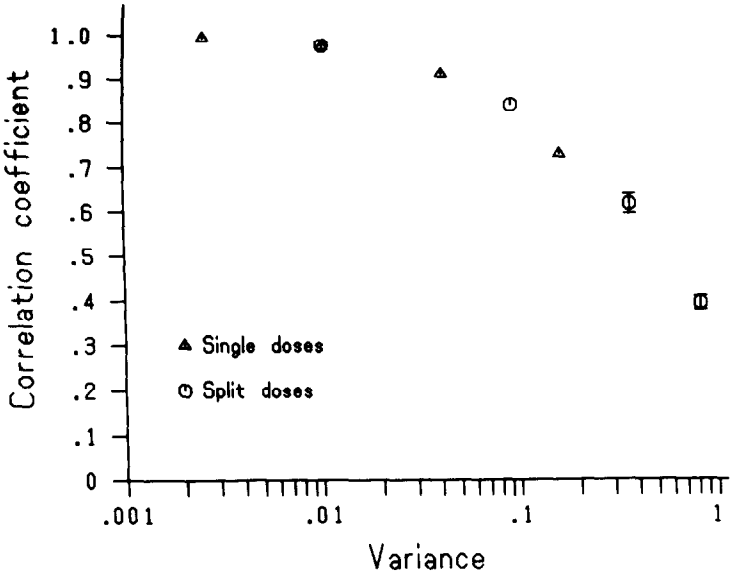

Fig. 5. The relationship, generated by Monte Carlo simulation of correlation coefficient for the dose–response relationship (5 dose-points for single treatments, 6 dose-points for split treatments) and the variance of the assumed underlying normal distribution of tumour cell sensitivity. The error bars are standard errors of the mean, generated by repeating the Monte Carlo simulation 5 times.

damage chosen as the end-point. Therefore, it is not easily related to the D_0 ratio estimates for cells in culture or for tumours *in vivo*. Consequently, it is perhaps premature to anticipate meaningful differences between normal and malignant tissues as to the development of thermotolerance *in vivo*.

With regard to thermotolerance in tumours, comparison of results is also complicated by differences as to 'priming' and subsequent treatments in different experiments. It is important, in order that a true thermotolerance be separable from repair of heat damage (which merely restores the original level of sensitivity), that the secondary heat treatments include at least some whose magnitude is greater than that of the first treatment [2].

In the present context, the results reported are most readily compared with those of Kamura *et al.* [16] for a mammary carcinoma implanted in the feet of CDF_1 mice. As in the present studies, these workers used a 'priming' dose of 30 min at 43.5°C, a variable time interval and a variable second treatment, also at 43.5°C, using re-growth delay as the tumour response end-point. Kamura *et al.* [16] found a maximum TTR of approximately 6 at 16 hr, falling to approximately 3.3 at 24 hr and 1.5 by 48 hr. By comparison, the thermotolerance ratios found here (Table 2) were 4.09 at 24 hr and 3.45 at 48 hr. The results at 24 hr are similar, though thermotolerance appears to fade more rapidly for the mouse mammary carcinoma than for the rat sarcoma.

However, in the present studies the development of thermotolerance appeared to be quite variable, the estimated 'D_0' distribution being considerably wider in the case of split-dose than of single-dose treatments. A similar effect is apparent in the data of Kamura *et al.* [16]. These observations imply that groups of tumours which respond similarly to a single heat treatment may respond differently to a second heat treatment. Apparently, the development of thermotolerance depends on factors other than those which govern response to a single treatment.

In summary, the development of thermotolerance by heated tumours appears to be a rather variable phenomenon which is capable of increasing the tumour cell 'D_0' by at least several-fold. It is of interest that since the tumour in the present study had occluded blood flow, transient hypoxia evidently does not preclude the development of a significant degree of thermotolerance by heated tumour cells. The broad similarity of the present findings (with blood flow occlusion) to those of Kamura *et al.* [16] (with blood flow unimpeded) could be interpreted as implying that occlusion *per se* and its interaction with hyperthermia does not affect development of thermotolerance, provided adequate heating can be achieved.

Evidently, more work is required on thermotolerance in both tumours and normal tissues before optimal schedules for hyperthermia treatment can be contemplated.

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APPENDIX

The approximately linear dose–response relationship observed in the present study may be represented by the simple equation:

$$\bar{\tau} = \bar{\tau}_0 + \lambda t, \quad (1)$$

where $\bar{\tau}$ is the mean cure-corrected re-growth delay, t the treatment time at 43.5°C, and λ and $\bar{\tau}_0$ are respectively the slope and the intercept of the best-fitting straight line. We assume that each individual tumour in the group conforms to such a linear relationship but that the individual sensitivities differ from tumour to tumour (intercept differences may be ignored in this context). Hence the ith tumour of a particular group conforms to the equation:

$$\tau_i = \bar{\tau}_0 + \lambda_i t, \quad (2)$$

with equation (1) resulting from plotting the average of the individual τ_is (to give $\bar{\tau}$) against time t. Variability in the λ_is will result in a poorer conformity of the data to equation (1), i.e. greater scatter in the data. Conversely, the quality of the linear relationship of equation (1) may provide guidance as to the variability of the λ_is.

In an attempt to quantify this variability we have made use of Monte Carlo simulation methods to explore the relationship between the variability of the λ_is and the quality of the linear dose–response relationships, as represented by the correlation coefficient of the best-fitted straight line. Monte Carlo methods, using computer-generated random numbers conforming to a specified distribution, have been employed to study the radiation res-

ponse of cells in culture [17], of the relationship of tumour cure to radiation dose [18], of tumour cure to regrowth delay [11] and of the factors controlling variability of response to experimental chemotherapy [19].

Here we employ Monte Carlo methods to examine the deterioration in the quality of a linear dose–response relationship as the variability of sensitivity to treatment increases. Random numbers conforming to a normal distribution with specified mean and variance were generated using a shift-register sequence generator. These random numbers, for a given variance of distribution, were taken to be the λ_is of equation (2) and a corresponding τ_i was calculated for each time point t of equation (1), and a best-line was fitted by the method of least squares to the resultant relationship of $\bar{\tau}$ to t. The correlation coefficient of the best line was recorded. The process was repeated five times to give a measure of the variability of the correlation coefficient. The mean and standard error of the correlation coefficient were calculated. The variance of the λ_i distribution was now increased and the entire process repeated.

In this way it is possible to obtain a plot of the correlation coefficient against the variance of the λ_i distribution. Such a plot is presented in Fig. 5, showing the progressive deterioration of the correlation coefficient as the λ_i variance (i.e. the heterogeneity of tumour cell sensitivity) increases. The data of Fig. 5 were used to obtain estimates of the variances of the underlying sensitivity distribution which would result in the correlation coefficients actually observed for the dose n response relationships in the three experimental groups. The implied sensitivity distributions are depicted in Fig. 4.