Intestinal crypt clonogens: a new interpretation of radiation survival curve shape and clonogenic cell number

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Abstract. Estimates of the clonogen content (number of microcolony-forming cells) of murine intestinal crypts using microcolony assays show an apparent dependence on the radiation dose used in the assay of clonogen content. Crypt radiation survival curves often show increased curvature beyond that expected on the basis of the conventional linear-quadratic model. A novel form of crypt survival curve shape is proposed based on two contributory mechanisms of crypt killing. Six previously published sets of microcolony data were re-analysed using a dual-kill model, where target cells are killed by two contributory mechanisms, each described by a linear-quadratic function of dose. The data were analysed as two series – high-dose rate and low-dose rate irradiation. The data were fitted to the models using direct maximization of a quasi-likelihood, explicitly allowing for overdispersion. The dual-kill model can reproduce both the apparent dose-dependence of the clonogen estimates and the high-dose curvature of the dose–response curves. For both series of data the model was a significantly better fit to the data than the standard linear-quadratic model, with no evidence of any systematic lack of fit. The parameters of the clonogenic cell component of the model are consistent with other studies that suggest a low clonogen number (somewhat less than five) per crypt. The model implies that there is a secondary mechanism decreasing clonogen survival, and hence increasing clonogen number estimates, at high doses. The mechanisms underlying the modification of the dose–response are unclear, and the implied mechanisms of, for example, slow growth, induced either directly in the surviving cells or indirectly through stromal injury or bystander effects are only speculative. Nevertheless, the model fits the data well, demonstrating that there is greater kill at high doses in these experimental series than would be expected from the conventional linear-quadratic model. This alternative model, or another model with similar behaviour, needs to be considered when analysing in detail and interpreting microcolony data as a function of dose. The implied low number of ≤ 5 of these regenerative and relatively radioresistant clonogenic cells is distinct from a similar number of much more radiosensitive precursor stem cells which undergo early apoptosis after doses around 1 Gy.

INTRODUCTION

The numbers of stem cells and clonogenic cells in the intestinal crypt are important parameters in the understanding the regulation of proliferation in normal cell replacement and the response to damage in the intestine. Mathematical modelling studies based on extensive cell-kinetic data indicate a number of around 4–6 stem cells per crypt (Potten & Loeffler 1990). The most direct measure of clonogen number is the microcolony assay (Withers & Elkind 1970) using a two-dose experimental design (Hendry 1979), where the number of regenerating crypts is assayed following a single dose or two doses of irradiation each the size of the single dose. However, it should be noted that in order to estimate clonogen numbers in this way, the system has to be perturbed which may itself alter the clonogen population. Equivalently, but rather less directly, the dose–response functions of crypt survival to fractionated radiation can be measured using the same assay. Then, appropriate radiobiological models can be fitted to the data and the clonogen number estimated as a model parameter (Thames & Withers 1980; Tucker *et al.* 1991). A third approach has been to use short-range electrons emitted from a Pm-147 plaque in apposition to the outside of the intestinal wall to irradiate different cell positions in the crypt with different doses (Hendry *et al.* 1989).

Unfortunately, these approaches have produced conflicting results, ranging from low clonogen numbers around 4–8 (e.g. Hendry *et al.* 1992), through to much larger numbers of the same order as the number of cells in the entire crypt (e.g. Withers *et al.* 1993a). In a series of experiments (Hendry *et al.* 1992; Roberts *et al.* 1995), we showed that the response to fractionated irradiation of murine intestinal crypts could not be explained by a simple mechanism in which crypts had a fixed number of clonogenic cells, and that the apparent number of clonogenic cells per crypt increased from around four when low doses were used in the assay, to 40 or more at higher doses. Potential explanations for this were that, at low doses the number of clonogens mimicked the number of steady-state stem cells, but at higher doses there might be greater recruitment of stem-cell daughters back into the stem-cell compartment. Alternatively, the higher doses produced greater levels of clonogen kill than predicted from the responses to lower doses, and this would result in higher calculated values of initial clonogen number. Indeed, in fitting many crypt survival curves over a number of years, we have observed that frequently there is a poor fit to the data at high doses, with a lower survival than expected at the top of the dose range (see for example Hendry *et al.* 2000).

To explore these differences further we undertook a series of experiments with 1–4 equal-sized fractions, analysed using both two-dose methodology and radiobiological models (Roberts *et al.* 1995). We showed again an apparent increase in clonogen number with the dose used in the assay. We investigated carefully the assumptions of the microcolony assay, and potential sources of error in the assay and in the analysis, but showed that these results were robust. Similarly, Tucker *et al.* (1991) carefully examined the effects of various forms of heterogeneity and found that their higher clonogen number of 168 was robust in the presence of heterogeneity.

In analysing the larger numbers of crypt survival curves arising from investigations on a variety of different strains of specific-gene-silenced mice (Hendry *et al.* 2000; Hendry 2002), and in other studies of radiation response modifiers, we have observed, as noted above, that in many cases the data at high doses do not fit the dose–response curve well, often with a greater than expected crypt kill at these doses. As the number of surviving crypts is generally low at these doses, the survival is not well-determined, and in any individual experiment such effects

are readily attributed to stochastic variation. In one published series using low-dose-rate (LDR) irradiation (Hendry *et al.* 2000), the high-dose portion of the crypt survival curve has been carefully determined, and the lack of fit of the data at > 30 Gy to the extrapolated fit from ≤ 30 Gy demonstrated, and verified by statistical analysis.

The apparent excess killing at high doses leading to a dose-dependence of the clonogen number suggests that the radiobiological models used to fit the crypt survival curves, and (directly or indirectly) to estimate the clonogen number, may be too simplistic. One possible alternative is to invoke a putative secondary mechanism of crypt sterilization to account for the excess crypt death at high doses, and this is the possibility investigated here. Whilst the model invoked has a clear mechanistic origin, such a model could also be interpreted more generally as an empirical modification that generates more killing at high doses, and thus be consistent with a far wider class of potential mechanisms. In this study we have re-examined the multifraction data (Hendry *et al.* 1992; Roberts & Potten 1994; Roberts *et al.* 1995) and the LDR data (Hendry *et al.* 2000) using a model for crypt survival involving two distinct mechanisms of crypt killing.

DATASETS AND METHODS

The microcolony assay

In the microcolony assay, the numbers of regenerating foci associated with intestinal crypts are scored in a fixed number of transverse gut sections following irradiation (Withers & Elkind 1970; Potten & Hendry 1985). Similar sections from unirradiated (control) animals provide estimates of the number of crypts present before treatment. The need for a correction for differences in the size of regenerating crypts when measured in longitudinal sections is well established (Potten *et al.* 1981; Potten & Hendry 1985) and such a correction has been applied to the datasets analysed here.

In the data analysed here, a series of equally sized fractions (up to four fractions) were used. These data can be analysed by fitting appropriate radiobiological models (see below). Alternatively, when there are data from one and two equal fractions, the data can be analysed as follows. The number of clonogens in the crypts, m , can be estimated from the number of crypts surviving one (S_1) and two equal-sized doses (S_2) from the following simultaneous equations (Hendry *et al.* 1992)

$$\begin{aligned} S_1 &= 1 - (1 - S_c)^m \\ S_2 &= 1 - (1 - S_c^2)^m \end{aligned} \quad (1)$$

where S_c is the clonogen surviving fraction after each dose. These can be simplified using a Poisson approximation to give the following form for the clonogen number:

$$m \approx \frac{-[\ln(1 - S_1)]^2}{\ln(1 - S_2)} \quad (2)$$

Equation 1 assumes simply that (i) there are a fixed number (m) of clonogenic cells per crypt, (ii) the two fractions kill clonogenic cells independently, both doses killing the same proportion of cells, and (iii) this is the only mechanism of crypt killing. Similar assumptions are implicit in the standard models of crypt survival. In our previous work we implicitly assumed that (ii) and (iii) held and thus had to contemplate the failure of assumption (i), that is, a non-constant clonogen number. Here, we explicitly investigate the consequences of relaxing assumption (iii),

which also implicitly invalidates assumption (ii), as the second mechanism of crypt killing produces a model in which multiple fractions do not act independently.

There are a number of technical problems with this assay, and these have been studied extensively (see for example Tucker *et al.* 1991; Hendry *et al.* 1992). In general these do not affect the clonogen estimates dramatically, with the exception of the need to use the full binomial model when the number of crypts killed is small (Hendry *et al.* 1992).

Fractionated high dose rate series

We used three sets of data obtained from experiments employing high dose-rate irradiation and multiple fractions of dose, which have been previously published and described in detail. These experiments were all performed using mice of the B6D2F₁ hybrid strain (BDF1).

1 The extensive series of two-fraction data with one or two equal-sized doses of ¹³⁷Cs γ -irradiation (Hendry *et al.* 1992). We refer to this series as 'series 1'.

2 A series of experiments designed to allow comparison of the two-dose methodology and the radiobiological modelling approach using data from 1 to 4 fractions of ¹³⁷Cs γ -irradiation (Roberts *et al.* 1995). We refer to this series as the 'four-dose' series.

3 A series of experiments in which we compared the usual microcolony assay based on gut sections with a variant assay based on measurements in intact epithelial sheets (Roberts & Potten 1994). These two methods gave very similar results and have been pooled for the present analyses. We will refer to this series as the 'sheets' series.

Low dose rate series

The work of (Hendry *et al.* 2000), which studied the effect of low dose rate ⁶⁰Co γ -irradiation (1 Gy per h) on p53 and bcl-2 knockout mice, provides good-quality single-fraction dose-response curves up to high doses and demonstrates well the phenomenon of excess kill at high doses. Here we utilize the data from mice of the three wild-type strains (hybrids in two cases) used in those experiments, namely C57BL, B2D6F₁, and 129Sv/C57BL6.

Model fitting and testing

We fitted a number of functions of the form $f(\theta; N, D)$ to crypt survival curves, where θ is a vector of unknown biological parameters, N is the number of fractions and D the total dose. One of the parameters in θ is the number of clonogens in the crypt, m . The crypt survival data are the number of regenerating crypts from a known initial number and as such will approximately follow a binomial distribution. The binomial assumption is an approximation as the number of crypts 'at risk' is itself a quantity estimated with uncertainty and which will vary by a small amount from histological section to section. It is also possible that the number of clonogens itself might vary from crypt to crypt. More significantly, there is clear overdispersion in the results among experiments, both between animals and dose points within an experiment and between different experiments.

The data were fitted to the models using direct maximization of the binomial quasi-likelihood (McCullach & Nelder 1989), the use of a quasi-likelihood takes into account the overdispersion in the data by inflating the binomial variance by a scaling factor. Ninety-five per cent confidence intervals were obtained using a profile-likelihood approach based on a quasi-likelihood F -test with the overdispersion parameter estimated using the Pearson statistic (Firth 1991). Models were compared using quasi-likelihood F -tests.

The models discussed here involve rather too many unknown parameters to be fitted reliably to the data from the six individual datasets within the two series. The parameter estimates are

highly correlated with each other. In order to obtain estimates, it is necessary to pool the data for the three datasets within each series. The difficulty in obtaining reliable fits to the more richly specified models makes traditional statistically determined model selection impractical. Hence we used a biologically based model selection procedure, fitting common estimates of parameters across series where this seemed biologically reasonable. These biological assumptions can be tested statistically using standard quasi-likelihood F -tests based on the deviances of the models compared with alternative models in which the assumptions have been relaxed.

The standard model

The conventional analysis of crypt radiation survival curves assumes that each crypt has m clonogens, each with a radiation sensitivity described by a linear-quadratic (LQ) function of dose. Thus after N equal-sized fractions of radiation the probability of a crypt surviving is given by the following function of the total dose, D

$$S(\alpha, \left(\frac{\beta}{\alpha}\right), m; N, D) = 1 - \left(1 - e^{-\alpha D(1 + (\frac{\beta}{\alpha}) \frac{D}{N})}\right)^m \quad (3)$$

Here, we have chosen to parameterize the LQ function in terms of the (β/α) ratio. As the clonogen number, m , must by definition be greater than zero, and by observation has a highly skewed distribution, it is preferable to express the model in terms of $\ln(m)$ and replace m in equation 1 by $\exp(\ln(m))$, fitting $\ln(m)$.

A dual-kill model

An alternative hypothesis can be based on the observed misfit between the data and the standard model at higher doses. From this we might infer that at these higher doses some crypts are being sterilized by a mechanism other than the direct elimination of the clonogens, and crypts that should have one or more surviving clonogens are failing nonetheless to form microcolonies. One interpretation of such a model would be that at high doses there are more small clones produced by fewer cellular divisions, or there is more induced differentiation of clonogens compared with their self-renewal, so that these clones are below the threshold for detection. Alternatively, there may be so much damage to the stroma in which the crypts are situated, that the crypts are unable to regenerate, even if they do have some clonogens which survived the direct radiation insult.

One simple way to represent such a phenomenon is to assume a biphasic survival function, B , with a secondary survival function of the same functional form as for the clonogens:

$$B(\alpha_c, \left(\frac{\beta}{\alpha}\right)_c, m_c, \alpha_s, \left(\frac{\beta}{\alpha}\right)_s, m_s; N, D) = S(\alpha_c, \left(\frac{\beta}{\alpha}\right)_c, m_c; N, D) \times S(\alpha_s, \left(\frac{\beta}{\alpha}\right)_s, m_s; N, D) \quad (4)$$

where α_c , β_c/α_c and m_c are the parameters of the clonogenic survival curve, whilst α_s , β_s/α_s and m_s are the parameters of the secondary survival curve. Clearly, if the interpretation of the second mode of crypt-killing is based on stromal effects, the parameters of the secondary survival function are unlikely to be readily interpreted directly as numbers and sensitivities of target cells, but rather as a convenient (and rather arbitrary) representation of the survival curve. For practical purposes, it will probably be sufficient to reduce this further to the two-parameter multitarget form by omitting the term in β_s/α_s . As there is no evidence for the tissue architecture of the target structures (if any) related to this second mechanism, and it is unlikely that it will prove

Figure 1. The crypt dose–response curves for two components (broken lines) and the resultant composite dose–response curve (solid line) of the dual-kill model (equation 4). The parameters used in this simulation are $\alpha_c = 0.05$ ($\beta/\alpha_c = 1/3$, $m_c = 5$, $\alpha_s = 0.2$, $(\beta/\alpha)_s = 1/10$, $m_s = 150$.

possible to get accurate data at these very low survival probabilities, this simple representation based on cell groups as targets is likely to be sufficient.

Figure 1 shows the form of equation 4 for a single dose, using parameter values similar to those obtained from the high-dose-rate (HDR) data (see below), with the two effects plotted separately. Here we see that, under the assumptions of the model, and with the parameter estimates chosen, the crypts are killed predominantly by loss of clonogens at lower doses, but that crypt loss due to the second mechanism is predominant at higher doses. The secondary effect becomes significant at rather modest levels of crypt kill, as the crypt surviving fraction drops below ~50%, well within the usual experimental range.

Using the model as defined in equation 4, we can compute crypt survival curves for single and double equal-sized doses and (from equation 1 or 2) the apparent number of clonogens that would be derived from a two-dose experiment. The results of such an exercise, using the radiobiological parameters of Fig. 1 (with 5 clonogens per crypt) is shown in Fig. 2. It is apparent that if this dual-kill model applies, then as the size of each dose increases, the apparent clonogen number increases from its ‘true’ value at low doses (five here) to unreasonably high numbers at high doses. Also shown in Fig. 2 is the difference between the binomial estimates of clonogen number (equation 2) and the Poisson approximation (equation 1), highlighting the need to use the more complex binomial form when the number of surviving crypts is near control levels.

The dual-kill model has six unknown parameters, and thus we need fairly extensive data, covering a wide range of dose and fraction numbers to be able to estimate the parameters reliably. Intuitively, one can see that we have a number of parameters that can reproduce a gentle downward curvature in the dose–response curve similar to that seen in the data. Multiple-dose fractionation can assist in the determination of the β/α , but we need good data at high doses (where the survival is smallest and the random errors in the data largest) to determine accurately the parameter values applicable to the proposed secondary mechanism.

Figure 2. Results of a two-dose experiment if the true crypt survival followed the dual-kill model (equation 4). The solid line is the estimate based on the full binomial computation (equation 1), whilst the dashed line shows the estimate using the less-accurate Poisson approximation (equation 2). Parameters as for Figure 1, with five clonogens assumed per crypt at zero dose. The horizontal dotted line represents an average total number of cells per crypt (250).

RESULTS

The standard model

Figure 3 shows the result of fitting this standard model to the multifraction series, where we have (unlike the final analysis in Roberts *et al.* 1995) been true to the spirit of the model and assumed the clonogen number, m , to be constant across all fractionation schemes. Table 1 gives the fitted parameters obtained. There is clearly a poor fit to the data in the four-dose series, which can be partly remedied by allowing the clonogen number to increase with increasing fractionation (Roberts *et al.* 1995). There is some evidence of a lack of fit at higher doses with the data points tending to lie below the fitted line.

Figure 4 shows the fitting of the same model to the LDR series, and Table 2 gives the parameter estimates. As there were only single-dose data, and hence very little information on the repair processes determining β/α , it is not possible to resolve all three parameters. However as the irradiation is given at low-dose rate it is reasonable to assume that the β/α term is negligibly small, and thus we fixed this term at zero and estimated the other parameters. These estimates differ slightly from the estimates of Hendry *et al.* (2000) as, here, the whole dataset was fitted, whereas the earlier work excluded data > 30 Gy from the fitting. In the present series, there is very clear evidence of a lack of fit at high doses. In the earlier work it was shown that the majority of data points > 30 Gy lay below the extrapolate of the fitted line from the data ≤ 30 Gy, falling outside the 95% confidence limits of the data (assuming a binomial distribution).

The dual-kill model

In order to estimate the parameters, we firstly pooled the data from the three multifraction studies, and secondly the three LDR strains. As there was some interstudy variation, we allowed the

Figure 3. The fit from the standard model to the multifraction series. Squares, single fraction; circles, two fractions; up-triangles, three fractions; down-triangles, four fractions. Each series is fitted as a separate dataset. The parameter estimates are given in Table 1. The two or four lines on each panel present the data for one, two, three and four fractions. The error bars represent the binomial 95% confidence limits of the data points.

Table 1. The fit from the standard model to the multifraction-dose series

Parameter	Dataset	Estimate	95% CI
α (Gy^{-1})	Series 1	0.10	0.04–0.15
	Four dose	0.08	0.05–0.12
	Sheets	0.12	0.04–0.19
β/α (Gy^{-1})	Series 1	0.20	0.09–0.58
	Four dose	0.21	0.13–0.39
	Sheets	0.14	0.06–0.54
m	Series 1	8.2	6.2–10.9
	Four dose	7.9	5.1–11.8
	Sheets	8.1	5.3–12.4

clonogen radiosensitivities α_c and α_s to take different values in each study/strain, but fitted common estimates for the two β/α parameters and the clonogen numbers. This choice of model can be justified post hoc (see below).

Table 3 shows the parameters estimated from the multifraction-dose series, and Fig. 5 shows the fitted functions. Table 4 and Fig. 6 show the parameters and the fit to the LDR series, where we again assumed that the two β/α parameters were zero at this low-dose rate.

Both datasets yielded similar estimates of clonogen sensitivity of 0.3–0.6 Gy^{-1} , and an estimate of $(\beta/\alpha)_c$ for the clonogens corresponding to an α/β ratio of around 3 Gy. The putative cells (or alternatively the unknown additional feature) associated with the second mechanism of crypt sterilization are rather more sensitive ($\alpha = 0.2$ –0.3 Gy^{-1}), and have a lower fractionation sensitivity [$(\alpha/\beta)_s \sim 10$ –50 Gy]. The multifraction series yielded a clonogen number estimate of ~ 5 (3–7), very much in line with the low-dose estimates from the same data (Hendry *et al.* 1992; Roberts & Potten 1994; Roberts *et al.* 1995), whilst the LDR series give a somewhat lower estimate of ~ 1 . With the wide confidence limits and the strong correlations

Figure 4. The fit from the standard model to the LDR series. Each series is fitted as a separate dataset. The parameter estimates are given in Table 2. The repair terms (β/α) have been set to zero as appropriate for low-dose-rate irradiation. The error bars represent the binomial 95% confidence limits of the data points.

Table 2. The fit from the standard model to the LDR series

Parameter	Dataset	Estimate	95% CI
α (Gy^{-1})	BDF1	0.14	0.09–0.19
	C57	0.16	0.05–0.31
	129	0.12	0.07–0.17
β/α (Gy^{-1})	BDF1	Fixed at zero	
	C57		
	129		
m	BDF1	7.2	2.6–19.5
	C57	10.7	0.7–207
	129	6.7	2.3–19.9

between the parameter estimates, these two estimates should not be considered significantly different.

A formal comparison using quasi-likelihood F -tests showed that the dual-kill model was a significantly better fit to the data than the standard model ($P < 0.0001$ for both series).

Fitting more compact models indicated that models in which the cell sensitivities (α_c, α_s) were assumed to take the same value in each dataset, were a significantly worse fit to the data ($P < 0.0001$ for both datasets). For the multifraction-dose series the two β/α estimates for the two mechanisms differed significantly ($P = 0.007$). The fits in Tables 3 and 4 did not differ significantly from a fit of a 'full' model in which all the parameters took different values in each series (multifraction: $P = 0.36$, LDR: $P = 0.12$), indicating that the model selected was an adequate and parsimonious fit to the data.

Based on this dual-kill model, we computed survival curves for one and two doses and, from these, computed the clonogen estimate that would be obtained from a two-dose experiment if this model applied. Figure 7 shows the estimated number corresponding to the survival curves of Fig. 3, along with the clonogen numbers directly estimated from the data. It is clear that a dual-kill mechanism such as that proposed could reproduce the apparent increase in clonogen number with dose observed in the split-dose experiments.

Figure 5. The fit from the dual-kill model to the multiple dose HDR series. Squares, single fraction; circles, two fractions; up-triangles, three fractions; down-triangles, four fractions. The error bars represent the binomial 95% confidence limits of the data points. A common value of the clonogen numbers, m_c and m_s , and the repair terms $(\beta/\alpha)_c$ and $(\beta/\alpha)_s$ have been assumed across the three series, with the cellular sensitivities α_c and α_s taking different values for the three series. See Table 3 for the parameter estimates.

Table 3. Parameter values from fitting the dual-kill model to the fractionated-dose series

Parameter	Series	Estimate	95% CI
α_c (Gy ⁻¹)	Series 1	0.060	0.007–0.095
	Four dose	0.056	0.008–0.086
	Sheets	0.060	0.013–0.094
$(\beta/\alpha)_c$ (Gy ⁻¹)	Series 1	0.28	0.16–3.2
	Four dose		
	Sheets		
m_c	Series 1	5.0	3.4–6.5
	Four dose		
	Sheets		
α_s (Gy ⁻¹)	Series 1	0.38	0.23–0.82
	Four dose	0.32	0.19–0.57
	Sheets	0.39	0.21–0.66
$(\beta/\alpha)_s$ (Gy ⁻¹)	Series 1	0.020	–0.040–0.085
	Four dose		
	Sheets		
m_s	Series 1	1330	127–90,100
	Four dose		
	Sheets		

DISCUSSION

The analysis presented above shows that the crypt microcolony data from the six studies analysed can be well fitted by a model incorporating two linear-quadratic (LQ) functions. Such a model fits the data significantly better than the conventional model in which crypt death is simply

Figure 6. The fit from the dual-kill model to the LDR series. Error bars are the binomial 95% confidence limits of the data points. The repair terms $(\beta/\alpha)_c$ and $(\beta/\alpha)_s$ have been set to zero as appropriate for low-dose-rate irradiation. A common value of the clonogen numbers, m_c and m_s , have been assumed across the three series, with the cellular sensitivities α_c and α_s taking different values for the three series. The parameter values are given in Table 4.

Table 4. Parameter values from fitting the dual-kill model to the LDR series

Parameter	Series	Estimate	95% CI
α_c (Gy ⁻¹)	BDF1	0.047	0.068–0.092
	C57	0.064	0.017–0.104
	129	0.032	0.004–0.073
$(\beta/\alpha)_c$ (Gy ⁻¹)	BDF1	Fixed at 0	
	C57		
	129		
m_c	BDF1	1.1	0.6–3.7
	C57		
	129		
α_s (Gy ⁻¹)	BDF1	0.24	0.18–0.37
	C57	0.21	0.15–0.35
	129	0.22	0.16–0.33
$(\beta/\alpha)_s$ (Gy ⁻¹)	BDF1	Fixed at 0	
	C57		
	129		
m_s	BDF1	245	54–13,300
	C57		
	129		

a consequence of clonogen depletion described by a single LQ function. This is in spite of the allowance made for the reduced sampling frequency of smaller foci based on their size (Potten *et al.* 1981). If the allowance was not made, the curvature in the survival curve would be even greater, because survival levels after lower doses would be spuriously higher and after higher doses survival would be lower. This new model reproduces the greater curvature of the single-dose crypt survival curve compared with that described by a single LQ function, as well as the apparent dependence of clonogen number on radiation dose. Other modifications to the single

Figure 7. Clonogen number as estimated by the split-dose methodology for the three multifraction series. The points represent the estimated clonogen numbers and their standard errors, and show clearly the apparent increase with dose. The lines show the number of clonogens that would be estimated from the fits of the dual-kill model to the data, in the absence of any recruitment, i.e. the fit of Table 3 and Figure 5.

LQ function may have fitted the data equally well, but the present method allows interpretation on the basis of several potential biological mechanisms. If we accept the possibility that there may be more than one mechanism of apparent crypt sterilization, then there is no need for a clonogen recruitment mechanism (Hendry *et al.* 1992) to explain the apparent increase in baseline clonogen content associated with an increase in the radiation dose used to elucidate this number.

It should be noted that any modification to the clonogen dose–response function alone would not be sufficient to reproduce the behaviour investigated here. As noted above, it is necessary to either introduce some second mechanism of crypt killing or introduce some other mechanism removing the independence of multiple fractions. A clonogen dose–response function that had a steeper slope at high doses (as observed in the observed single fraction data), but maintained the independence of multiple fractions, would not be sufficient. One possible alternative interpretation of the dual-kill model would be to see it as a mechanism (albeit a complex one) for introducing a correlation between multiple fractions.

In its derivation, the model implies two killing mechanisms, one related to the conventional stem-cell depletion, and the other as damage to some secondary structure. The first mechanism is well established, and the clonogen numbers and sensitivities are in line with that expected of crypt progenitor cells. It is possible that the crypts modelled as killed by this second mechanism represent very slow-growing colonies that fail to reach a detectable size by the time of the assay (3–4 days). Indeed, when smaller foci of five cells or fewer were included in the microcolony scores, this produced a less steep and less curvy single-dose survival curve. The latter implied a lower number of initial clonogens per crypt, which was also found (by about 2-fold) when higher doses were used with the two-dose technique (Hendry 1993). Also, after 14 Gy, the numbers of surviving crypts did increase by 7–20-fold when assayed at day 7 compared with day 3,

but the effect of this on deduced clonogen number was not assessed (Chomiczewski *et al.* 1993). A dose-dependence of this increase would reduce the influence of this effect on deduced clonogen number. The small colony effect has also been discussed in some detail in the context of large-intestinal crypts (Cai *et al.* 1997) where, in this site, little difference was found between colonic crypt survival curves based on thresholds between four and 10 cells. This slow growth effect has been well documented in cell-culture studies using established cell lines (Nias 1968). Nonetheless, this slow growth *in vivo* could be in part a consequence of damage to the supporting stromal environment, as noted for example with haemopoietic stem-cell regeneration in repeatedly irradiated bone marrow (Hendry & Lajtha 1972).

It is plausible that the death of another population of cells in the surrounding stromal environment of the crypt could lead to the death of the crypt – for example by disruption of nutrient or oxygen supply, or by the removal of survival or clonogenicity factors. The nature of early damage to the intestinal stroma affecting crypt-cell responses following irradiation is controversial. Paris *et al.* (2001) have reported in their mouse strains that there is severe damage to the vasculature demonstrated by apoptosis of endothelial cells, and they claim that this is the principle mechanism of crypt killing in their mice. These data and their interpretation have been disputed (Hendry, Booth & Potten 2001; Suit & Withers 2001), and the findings have yet to be corroborated by others. The first events after irradiation have been reported, in contrast by others, to be apoptosis in the crypt stem-cell zone within 2–3 h of irradiation (Potten 1977; Potten & Grant 1998). The presence of extensive endothelial cell apoptosis should produce widespread acute haemorrhaging and subsequent hypoxia to an extent not generally reported (Suit & Withers 2001). However, these data and the attendant controversy, and the haemopoietic findings above, indicate that it might be possible to reduce the proliferative/renewal capacity of irradiated clonogens by a stromally induced mechanism along the lines implied in the model, and that different mouse strains and/or different experimental conditions might influence the relative importance of two contributory mechanisms to crypt killing. There are other observations *in vivo*, where damage to the vasculature/stroma causes slower re-population of tumours after irradiation (the well-known tumour-bed effect), but this was considered largely to be the result of prevention of endothelial cell growth after irradiation, rather than the killing of existing endothelial cells (Brown 1987). Again, in contrast, there is a recent report that tumour response to radiotherapy may be regulated by radiation-induced endothelial cell apoptosis (Garcia-Barros *et al.* 2003).

An alternative target for the secondary ‘killing’ mechanism might be the intestinal neural elements which are clearly important in regulating the behaviour of crypt epithelial progenitors during cell replacement and regeneration (Bjerknes & Cheng 2001). Damage to these elements by higher doses of radiation might impair clonogenic regeneration.

The parameter values involved in the secondary mechanism as deduced from the fitting are difficult to interpret. Do the values give a clue about potential mechanisms? For the clonogens, $\alpha = 0.3\text{--}0.6\text{ Gy}^{-1}$, and $\alpha/\beta = 3\text{ Gy}$. The former value is consistent with other previous estimates for intestinal clonogens, the latter is in agreement with the value of 3.6 Gy previously estimated for clonogens in steady state, i.e. assessed using single and paired doses (Hendry & Potten 1988). For fractionated doses when clonogens are cycling more rapidly during the fractionation schedule, the α/β -value is higher at around 13 Gy (Thames *et al.* 1981). For the second mechanism, the α/β -value is much higher. Although this is not characteristic of stromal reactions, which generally are late reactions and which show low α/β -values, the response is somewhat more sensitive (higher α value). Hence, the two parameter values describing the second mechanism are characteristic of a response sensitive to dose but not to dose fractionation. This is indeed the case also for crypt cell apoptosis after low doses, which is very radiosensitive but dose-rate independent (Hendry *et al.* 1982), but in the present case the target is much more resistant than is

the case for apoptosis. Also, the curve for these targets has a large shoulder (Fig. 1), indicating that either many targets need to be killed to cause significant additional damage to the crypt, or that there is just a large threshold dose for the effect.

It should also be noted that various factors are known to change the shape of the survival curve. Some factors tend to make the curve 'pivot' about a point near the top of the curve, e.g. circadian variations (Hendry 1975), topographic differences in intestinal site (Moore & Maunda 1983; Firth 1991), previous irradiation of the intestine (Potten & Hendry 1983), whereas other factors tend to shift the whole curve, e.g. exogenous interleukin-1 (Hancock *et al.* 1991), prostaglandins, growth factors and cytokines (Booth & Potten 2001) or chemical radioprotectors (Hanson *et al.* 1988). Hence, there are some factors known to contribute to the shape of the whole curve, and others which affect just the high dose portion. These datasets have not been analysed in the present way to detect the presence or absence of the second component to the single-dose survival curve. This evidence could be interpreted on the basis of any of the mechanisms described above. Additional mechanisms can be envisaged, for example, unknown bystander effects such as the possibility that increasing damage to either the epithelium or the stroma as the dose increases could lead to the release of factors which increase the radiosensitivity of the remaining clonogens. There is some evidence that the first few apoptoses generated at low doses, which are manifest at 2–3 h post-irradiation (Potten 1977) release signals that initiate a regenerative round of cell proliferation in surviving clonogens at a time when there are no other detectable effects in the crypt cell population (Hanson *et al.* 1980; Hanson & Boston 1983; Potten *et al.* 1984; Tsubouchi & Potten 1985; Potten 1991a,b). It is possible that at high doses, when more and more cells are reproductively sterilized (but not induced into apoptosis), cytotoxic signals are released that increase clonogen sensitivity or reduce the time course of clonogen regeneration (a bystander effect).

There is other evidence which points to slower re-population as a likely mechanism. It was shown in earlier studies that, although curvature at high dose was found in the microcolony data, the macrocolony data obtained after even higher doses tended to follow the extrapolation of the lower-dose portion of the microcolony curve, rather than follow the curving high-dose portion (Withers & Elkind 1970). In the macrocolony assay, an additional 7 days over the 3–4 days for the microcolony assay is allowed, by which time small colonies presumably have had more time to regenerate and form visible nodules. In the light of all these considerations, and the lack of additional evidence regarding mechanism, we consider that the parameters calculated here should be regarded at present as a simple empirical description of the secondary curve.

The model used here suggests that the most representative clonogen number in the murine small intestinal crypts in the strains used in our studies is in the range of 1–5, and probably the best estimate is in the middle to upper part of this range. This value is consistent with our own data using short-range β -irradiation (Hendry *et al.* 1989), and with stem-cell numbers deduced from cell-kinetic modelling (Potten & Loeffler 1990). The similarity in low clonogen and stem-cell numbers may be fortuitous. In strains of mice where specific genes involved in apoptosis were silenced, changes in radiation-induced apoptosis in the stem-cell zone did not directly track clonogen radiosensitivity changes (Hendry 2002). Also, although a dose of 1 Gy causes apoptosis in four cells in the stem-cell zone (Potten 1977; Potten & Hendry 1983; Potten & Grant 1998), this dose does not sterilize a significant number of crypts. The majority of clonogens are more radioresistant and probably die post-mitotically. This could imply that the clonogenic population is heterogeneous in radiosensitivity, comprising a few very radiosensitive stem cells and a few more-resistant clonogenic non-stem cells, as discussed earlier (Hendry *et al.* 1982). Such heterogeneity is the case in short- and long-cycling stem spermatogonia (van der Meer *et al.* 1993), and to some extent in the haemopoietic cell hierarchy (Down *et al.* 1995).

Others have found a much larger number of apparent clonogens in their mouse strains and their experimental conditions, up to improbably large numbers of the order of the number of cells in the crypt (e.g. Thames *et al.* 1981; Withers *et al.* 1993a). Withers *et al.* (1993a) calculated a value of 123 (95% confidence interval: 108–140) clonogens per crypt from multifraction irradiation responses, and a lower dose-dependent range of 26–82 when the paired-dose technique was used. There are several potential technical reasons for the apparent differences in calculated clonogen numbers between investigators, apart from influences related to the modifying factors described above. These include, variously, the lack of use of a crypt size correction to take into account the different sampling frequencies of regenerating versus control crypts in sectioned material (Potten *et al.* 1981), differences in scoring criteria (Hendry 1993), and perceived greater difficulties in scoring control than irradiated sections (Withers *et al.* 1993b). The dual-kill model proposed here offers another possible explanation for this discrepancy: if there are two contributory mechanisms of crypt killing, the relative importance of the two might vary between strains and possibly be dependent on experimental procedures and animal husbandry (diet, gut microfauna, microbiological status, etc.). At higher doses and higher numbers of dose fractions, a microcolony assay might be largely governed by the secondary component. The cell-number parameter for the secondary component (m_s) estimated here is consistent with the high estimates of clonogen number seen in other studies and discussed above. It would be useful to explore the second component in the model in more detail using mouse strains where particular genes involved in epithelial and endothelial cell apoptosis and repair are silenced.

We have presented a model which accounts better for the observed microcolony survival data after irradiation in our studies, than does a single linear-quadratic function. However, the model is rather over-parameterized in terms of the available data, and it is difficult to fit to the data. This difficulty is a structural feature of the model, with several biological phenomena having similar effects on the resultant crypt survival function. In order to get reliable estimates of the model parameters, we have had to use data from multiple experimental series. Unless one can obtain data that cover the dose regimens in which both the cell-kill mechanisms operate, it will not be practical to fit the full dual-kill model to most experimental datasets. The model of equation 4 is not readily simplified, other than by the use of low-dose rate to eliminate the beta term. In view of the complexity of the crypt dose–response curve and the difficulty of determining the contributions of the different effects to the curvature in that curve, it seems as if the simpler split-dose approach might be more useful, covering a range of doses, and interpreting the low-dose limit as better representing the clonogen responses.

Throughout the derivation and analysis presented here, we have explicitly identified the first component of crypt killing with the clonogenic cells, and the second component as a secondary mechanism. Given the other evidence on clonogen number and the results obtained by the Manchester group, this is a logical association. However, other explanations are possible, as discussed above.

We have demonstrated that the inclusion of a second killing mechanism can account for the shape of the crypt survival curves at higher doses, and for the apparent increase in clonogen number with dose. This model, or other similarly extended models are required to fit and interpret crypt survival curves over the full dose range.

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