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Clonogenic response of cells of murine intestinal crypts to 12 cytotoxic drugs

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Summary. *The in situ response of clonogenic cells in murine intestinal crypts to graded doses of 12 clinically used cytotoxic drugs has been measured by the crypt microcolony assay. For the alkylating agents mechlorethamine hydrochloride and BCNU, survival curves down to three logs of crypt depletion could be obtained. However, with isopropyl methane sulphonate only one log of depletion was measurable, and for a fourth alkylating agent, cyclophosphamide, no crypt killing occurred before other toxicities resulted in death of the animal in less than 3–4 days. This is the major constraint on the use of the microcolony assay with cytotoxic drugs. Of four antibiotics, survival curves were obtainable for adriamycin, bleomycin and mitomycin C, but not for actinomycin D. 5-Fluorouracil gave only a limited degree of crypt kill. Of three S-phase-specific drugs, hydroxyurea and vincristine resulted in little or no crypt depletion, but there was dose-dependent crypt depletion after methotrexate. The complexities of applying cellular-kinetic classifications of cytotoxic drugs to in situ endpoints are discussed.*

Introduction

There is an extensive literature on the clonogenic response of murine intestine to cytotoxic drugs in combination with ionising radiation (reviewed recently by Phillips [11]). In these experiments, the cytotoxic drugs have been used in two main contexts: (i) toxicity studies, where interest centres around the influence of a given drug on damage to the intestinal mucosa, a potentially dose-limiting tissue in clinical therapy; and (ii) fundamental studies, where particular biological effects of a drug, known from other cells or tissue systems, are utilised to investigate the nature of the clonogenic cells in untreated or irradiated intestine. An example is the use of hydroxyurea or suicide doses of tritiated thymidine, which are assumed to kill only the S-phase compartment of the clonogenic cell population [2, 4].

In either kind of investigation, it is difficult to separate entirely satisfactorily the individual effects of the two modalities. Shifts in radiation dose-survival curves after pretreatment with drug are frequently interpreted in terms of alterations to the radiation sensitivity of the cells, such as reduction in the capacity to repair sublethal radiation injury (e.g., [6]). However, the major property of these drugs is that they kill cells. We demonstrate here that dose-survival curves for intestinal clonogenic cells can be constructed for drugs of different chemotherapeutic classes given alone; indicate some of the technical difficulties; and examine the complexities of using these drugs to infer the kinetic status of target cells in the intestinal mucosa.

Materials and methods

Mice. Male, first-generation hybrids of the C57BL/6 × DBA/2 cross ($B6D2F_1$) were obtained from the Paterson Laboratories' breeding colony. Experimental animals were used at 9–11 weeks of age, when their mean weight was 25 g (2 SD = 3 g). The mice were kept under a 12-h dark (18:00–06:00), 12-h light regimen, housed in boxes of five, and allowed standard mouse diet (Labsure, Poole) and tap water ad libitum.

Drugs. Unless otherwise indicated, all drugs were dissolved and diluted in sterile, 0.9% saline. The drugs used were: Mechlorethamine hydrochloride (HN-2; Boots, Nottingham); 1,3-bis (2-chloroethyl)-1-nitrosourea (BCNU; Bristol Labs, Syracuse) dissolved in absolute ethanol and diluted in saline; isopropyl methane sulphonate (IMS; Koch-Light, Colnbrook); cyclophosphamide (CY; Ward Blenkinsop, Wembley); adriamycin (ADR; Montedison, Barnet); bleomycin (BLM; Lundbeck, Luton); mitomycin C (MIT C; Kyowa, Tokyo); actinomycin D (ACT D; Merck Sharp, Rahway); methotrexate (MTX; Lederle, Gosport); 5-fluorouracil (5FU; Roche, Welwyn Garden City); vincristine (VCR; Lilly, Basingstoke); and hydroxyurea (HU; Squibb, Hounslow). The injection volume was 0.4–0.5 ml in all cases. Drugs were injected IP as single doses given at 09:00–10:00 h, except for HU, which was injected in four doses each separated by 2 h, either between 10:00 and 16:00 h or between 22:00 h and 04:00 h.

Assay. The effect of treatment on survival of whole crypts of the jejunum was measured by the microcolony assay described by Withers and Elkind [14]. Four to six mice were used per experimental point. Animals were killed 4 days after treatment. This interval was chosen as being at or near the peak of mitotic recovery in crypts after these drugs [8]. The jejunum was removed and 5-μm-thick transverse sections stained with haematoxylin were prepared. The number of regenerating crypts (i.e., foci of non-Paneth cells containing more than 20 cells) per gut circumference was scored for 12–30 circumfer-

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ences per dose per experiment. A surviving fraction (SF) of crypts was calculated relative to the number (121 ± 3) around a circumference in saline-treated controls. Results were confirmed by at least one repeat experiment, the data being pooled. The probability of transecting a crypt in a section of fixed thickness is proportional to the diameter of the crypt in the longitudinal plane of the gut. This influences the apparent SF of crypts. A correction factor has been applied to accommodate any differences in crypt size [3]. For each dose group the maximum crypt diameter in the transverse plane was measured and an average value was obtained for 15 crypts. Then the final SF is given by: raw SF × (average diameter in control crypts/average diameter in treated crypts). This correction factor assumes that crypt diameter is the same in any plane, which is a reasonable approximation in the case of regenerating crypts.

Analysis of dose-response curves. Objective curves were fitted to the data by a computer program, using a model for cell killing [1] which assumes that clonogenic ('structure-rescuing') cells in a multicellular structure behave in accordance with Poisson statistics, i.e., that the structure remains intact until, on average, fewer than three cells survive per structure; that survival of *cells* is exponential over the range of doses being analysed; and that the structure may regrow from one or more surviving cells. Optimum fits were obtained by minimum-chi-square methods, with each dose-point being assigned a 'weight' inversely related to the expected variance on that point [1]. Two parameters were calculated from the fitted curves: (i) D_0, the reciprocal of the final slope of each curve; and (ii) LD_{37}, the dose required to reduce the population of *crypts* to an SF of 0.63 and which is also the dose that, on the basis of Poisson statistics, would be expected to leave on average one surviving clonogenic cell per crypt [12]. From the values of LD_{37} and D_0 an 'extrapolation number' can be calculated [1]. This number, exp $[LD_{37}/D_0)$ equals (NαE), where N is the number of cells at risk in the crypt, α is the probability that one such cell can reconstitute a crypt, and E is the extrapolation number of each such cell.

Results

The crypt microcolony assay for the effect of cytotoxic drugs requires that the animals remain alive for 3–4 days after treatment. We have shown elsewhere that while certain drugs permit this period of survival over a wide dose range, in other cases time of death is a strong inverse function of dose [8]. For the 12 drugs we have examined here, the practical upper limit of dose in each case was the $LD_{75/4}$ i.e., the dose that killed 75% of mice within 4 days. This consideration effectively determined the length of crypt survival curve that was obtainable for the different drugs.

Figure 1 shows the clonogenic response of intestinal crypts to four alkylating agents. For both HN-2 and BCNU, curves for crypt survival spanned three logs, a result comparable to that after radiation. The value of $\exp(LD_{37}D_0) = (N\alpha E)$ for BCNU was 60-fold higher than that for HN-2. For the monofunctional alkylating agent IMS, a crypt survival curve of less than one log of depopulation was obtainable. This drug contrasts with HN-2, BCNU, and radiation in that as the dose is increased so the proliferative 'rebound' of surviving crypt cells is lessened or progressively delayed. The average crypt diameter in untreated crypts was 36 μm. Four days after

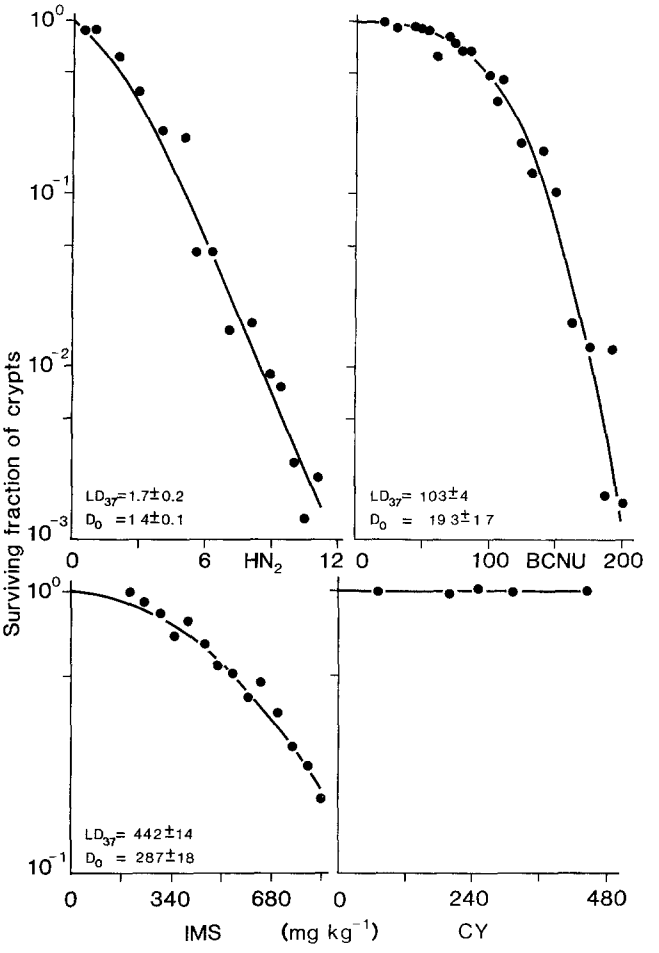

Fig. 1. Surviving fraction of crypts 4 days after single IP doses of four alkylating agtents

treatment the *average* diameter was 49 μm with HN-2, for BCNU 45 μm, and for IMS only 34 μm. With CY, no crypts were ablated at single doses up to 450 mg/kg. Survival time of the animal after CY is a particularly steep function of dose [8], so there was no opportunity to test directly whether this absence of crypt killing represented a very long threshold region prior to a steep fall in survival, as is seen for radiation.

The response of crypts to four antibiotics is shown in Fig. 2. In general, reproducibility of results was less good with these drugs than with the alkylating agents, despite identical experimental techniques. Each datum point for ADR represents on average the result of six separate experiments (range 2–23 experiments). Marked variation was observed in crypt survival between different gut circumferences from individual animals, suggesting inhomogeneities in distribution of the drug, possibly associated with use of the standard experimental route of injection, IP. The data for BLM were similarly scattered, but were of interest because of the absence of an initial shoulder and the suggestion that the curve might have a resistant tail. The MIT C curve was shouldered, with (NαE) = 10. ACT D did not destroy whole crypts at doses up to 0.9 mg/kg.

Survival of crypts after 5FU was a shallow, inverse function of drug dose, as was that after MTX, for which reproducibility was particularly poor (Fig. 3). In both cases, the average crypt diameter tended to fall at higher drug doses.

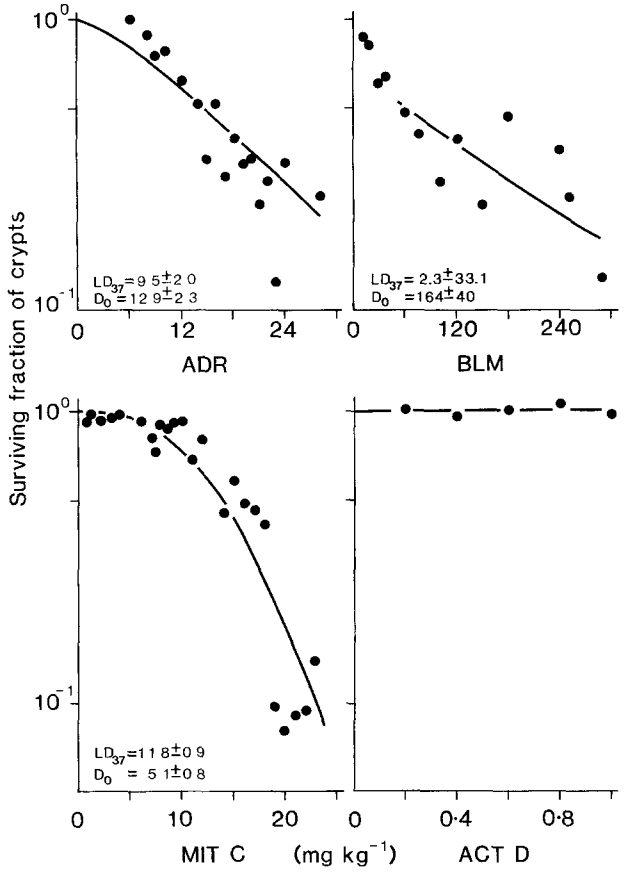

Fig. 2. Surviving fraction of crypts 4 days after single IP doses of four antibiotics

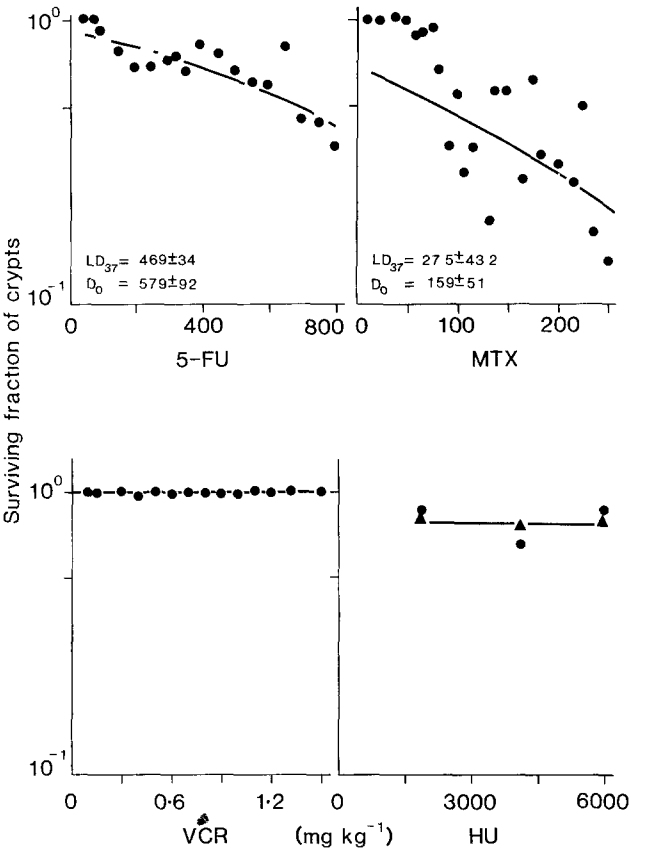

Fig. 3. Surviving fraction of crypts 4 days after IP treatment with four cycle- or phase-specific drugs. Four doses of HU were injected, at 2-h intervals, during either the light (●) or the dark (▲) period of the diurnal cycle

This small size (average crypt diameter 38 μm), together with progressively weaker staining with haematoxylin, meant that scoring of the response to these drugs was technically difficult. No crypts were killed by VCR with single doses up to 1.5 mg/kg. For HU, four repeated doses were used to maintain high drug concentrations during the middle of either the light or the dark periods of the animals' diurnal cycle. A small depletion of crypts occurred at both these times, with no dose-dependence of effect over the range measured.

Discussion

A mathematical model that describes the clonogenic response of whole crypts and their constituent cells in irradiated intestine [1, 12] fitted the data for HN-2 and BCNU well over a long range of survival (Fig. 1). The data for IMS, MIT C, 5FU (and less certainly, ADR and MTX) also conformed to the expectations from the model and the postulated cellular organisation of the crypt, i.e., progressively steeper slope over the first log of crypt deletion. With the exception of BCNU, values of (NαE) were very low, ranging from 1 (BLM) to 10 (MIT C). This contrasts with radiations such as ^{137}Cs gamma rays, where (NαE) for these B6D2F$_1$ male mice was 667 and the calculated value for N alone was 54 [10]. Possible reasons for this unresolved discrepancy have been rehearsed in greater detail elsewhere [7]. Briefly, either (i) the crypt cell population(s) at risk to different agents are qualitatively and/or quantitatively different; or (ii) the *administered* dose of drug, which is almost universally used as the independent variable of dose-response curves, is an inadequate descriptor of the drug concentration-time product that actually determines cytotoxicity in individual tissues.

If the values of (NαE) do represent an upper limit to the number of cells at risk per crypt, then plainly only one or two D_0's-worth of drug are required to reduce the cell population to a level where crypts are destroyed. For many drugs, depletion of the crypt population within the SF range 0.9 to 0.1 is sufficient to kill the animal within 8 days of treatment. This time-scale is commonly believed to be associated with death of the animal through a gastrointestinal syndrome (although it is unlikely that this is the cause of early death after all drugs [8]).

Low values of (NαE) for crypts become important in a therapeutic context when the D_0's for crypt cells are comparable to those for the classically dose-limiting tissue in chemotherapy, the haematopoietic tissue, because threshold doses for expression of injury and lethality doses are then more closely similar than for, say, whole-body irradiation. Lohrmann and Schreml [5] provide comprehensive lists of surviving fraction versus single dose of drug for the primitive, pluripotent cells of the murine bone marrow (CFUs) and the committed granulocyte-macrophage progenitors (GM-CFC) for a number of the drugs that we have used here. We have analysed these curves for single-cell survival 16–24 h after a single IP injection of the various drugs. Data were taken from several studies and for different strains of mice, so that the calculated D_0's represent average values (Table 1). Meaningful comparisons could be made between D_0's for crypt cells

Table 1. Mean D_0 values of dose-survival curves of cells of murine intestinal crypts, and for bone-marrow CFUs and GM-CFC assayed 16–24 h after IP injection of cytotoxic drugs (marrow values analysed from data in [5])

	D_0 (mg/kg)		
Drug	Crypt cells	CFUs	GM-CFC
HN_2	1.4	3.2 (5)	1.4 (1)[a]
BCNU	19	29 (3)	17 (1)
IMS	287	ND	ND[b]
CY	550[c]	101 (12)	150 (4)
ADR	13	15 (4)	ND
BLM	164	375 (2)	ND
MIT C	5.1	ND	ND
ACT D	–	0.51 (3)	ND
5FU	579	70 (3)	36 (4)
MTX	159	354 (5)	1520 (6)
VCR	–	ND	ND
HU	Plateau at SF = 0.8 (X 4 × 2 h) intervals	Plateau at SF = 0.5 (5) (X 1)	ND

[a] Figures in brackets are the number of studies from which data were analysed
[b] No data, or insufficient for analysis
[c] Inferred from data for drugs plus radiation [9]

(present study) and one or both of the haematopoietic cell types for seven drugs. Of these, HN-2, BCNU, ADR, BLM, and MTX revealed equal or lower D_0's for crypts than for the marrow cells. 5-Fluorouracil, commonly used in the treatment of bowel tumours, yielded a much higher D_0 for crypt cells than for either of the marrow cells. Cyclophosphamide does not ablate crypts, which could imply either a very high value of ($N\alpha E$) or a very high D_0. From data on the combination of graded doses of CY plus a test dose of radiation, we have calculated a high D_0 of 550 mg/kg for this drug [9]. Cytotoxic drugs have been suggested as alternatives or adjuvants to radiation for marrow ablation in the therapy of leukaemia. If man were to behave as do these mice, then although some drugs (e.g., CY), would have a large safety margin for gut damage, others (e.g., HN-2) might cause acute gut injury at marrow-ablating doses.

Like CY, ACT D and VCR failed to ablate crypts, and repeated doses of HU produced only a small depletion. The cytotoxic effect of ACT D is cell-cycle-specific and at lower doses may be S-phase-specific, while VCR and HU are regarded as S-phase-specific (various authors, cited by Lohrmann and Schreml [5]). Previous studies on $B6D2F_1$ mice, in which suicide doses of tritiated thymidine plus test doses of radiation were used, suggested that the great majority of intestinal clonogenic (microcolony-forming) cells were outside the cell cycle or at least not in the S-phase during the middle of the light period of the animals' diurnal cycle [4]. It was also inferred that many of these cells entered the S-phase during the middle of the dark period. Given these observations, the failure of single doses of VCR and ACT D to ablate crypts when given during the light period was not unexpected. Similarly, the dose-independence of the small effect of repeated doses of HU was compatible with survival of a population of drug-resistant (non-S-phase) cells, the effect saturating at the lowest dose used here. However, the equal crypt depletion after HU given in the middle of the dark and light periods differs from the results obtained with tritiated thymidine plus radiation, and this difference remains unexplained. Moreover, while VCR and ACT D had no overt effect, a third S-phase-specific drug, MTX, did ablate crypts in a dose-dependent manner down to a SF of 0.2 (Fig. 3). These last data mentioned illustrate potential pitfalls of assuming that the use of self-limiting, S-phase-specific drugs will yield 'absolute' values for the proportion of cells that are in S-phase in an in situ population. The value of ($N\alpha E$) for MTX was only 1.2. If for present purposes we take this to mean that each crypt contains only about one clonogenic cell, then use of a dose of 100 mg/kg would lead to the conclusion that 100% minus 46% = 54% of crypts/cells were in S at the time of treatment, but use of 250 mg/kg would indicate that 100% minus 22% = 78% were in S (Fig. 3). For in situ studies like those described here, where considerable time elapses before assay, the response to a given drug is the resultant of its half-life in the particular tissue and the nature of the cell population at risk during that time. It has been inferred that noncycling clonogenic cells of the murine intestine may be recruited into cycle within a few hours of a first cytotoxic insult by radiation (e.g., [4] or HU [2]. Hydroxyurea has a half-life of approximately 30 min in mice (average of two values quoted in [5]), but that of MTX appears to be several hours [13]. In the latter case, the opportunity for recruitment of target cells into a drug-sensitive phase is much greater.

Cellular-kinetic classifications of drug effect are unlikely to be wholly adequate in predicting the response of cells in situ in a tissue, exemplified here by the marked resistance of the crypt clonogenic cells to CY, for as yet unknown anatomical, pharmacokinetic, or biochemical reasons. However, we have demonstrated that clonogenic cells in this acutely responding tissue were sensitive to the lethal action of a number of drugs when the tissue had not previously been perturbed and when the majority of cells were believed not to be in cell cycle [4]. In the clinical situation, drug treatments are seldom given singly, and if quiescent, intestinal clonogenic cells are recruited into cycle by prior treatment, then the values for sensitivity that we have obtained here are likely to be upper rather than lower limits.

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