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Do cells of continually renewing populations and those stimulated from quiescence respond similarly to HU and Ara-C?

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Summary. *The cytocidal and cytostatic effects of a single dose of either hydroxyurea (HU) or cytosine arabinoside (Ara-C) have been studied in a variety of tissues, from both rats and mice, by autoradiographic and histological methods.*

The necrogenic effects of the drugs on DNA-synthesising cells from continually renewing populations have been compared with the effects on cells induced to enter DNA synthesis from a formerly quiescent state. Four models of stimulated proliferation have been studied; the isoprenaline-stimulated salivary glands, the testosterone-stimulated seminal vesicle in the castrate, the regenerating liver after two-thirds partial hepatectomy, and a model of diethylnitrosamine (DEN)-induced hepatocyte proliferation.

The time course of the cytokinetic response in each tissue was followed for a period of 24 h after drug treatment. The cytostatic effects of the drugs were similar in all tissues investigated, DNA synthesis being inhibited for a 4- to 5-h period. However, the sensitivity to the lethal effects showed both tissue and species variability.

Introduction

There is growing evidence that biochemical differences exist between continually dividing cells in the G_1 phase of the cycle and proliferatively quiescent cells in a putative G_0 phase. Darzynkiewicz et al. [6] have shown that the chromatin packing of resting lymphocytes differs from that in lymphocytes in the G_1 phase. Recent work [24] demonstrates that the histone synthesis in quiescent Chinese hamster ovary cells varies from that in cycling cells.

Farber and Baserga [10], on the basis of studies with HU, have proposed that cells recruited into DNA synthesis from G_0 show higher resistance to the cytocidal effects of HU than do cells entering DNA synthesis from the G_1 phase of the cycle. They postulated that this was due to a biochemical difference between G_1 and G_0 cells which, if correct, could be exploited in cancer chemotherapy and in studies to elucidate the mechanisms by which these drugs cause cell death.

Therapeutic perturbation of a tumour results in cells undergoing redistribution within the phases of the cell cycle and entry of formerly non-proliferating cells into a proliferative compartment [22]. Some treatment schedules involve hormone manipulation to stimulate tumour cells to enter the cell cycle prior to chemotherapy. For example [17], androgen priming has been used in attempts to enhance cell kill in human prostate tumours, on the assumption that cytotoxic drugs are most effective against actively proliferating cells. In these situations many of the cells in the proliferative compartment at the time of tumour therapy have been recruited from a non-proliferating population. The notion put forward by Farber and Baserga implies that these recruited cells may be relatively insensitive to drug treatment.

We have therefore further investigated the hypothesis that the recruited cells have a decreased sensitivity to the cytotoxic actions of HU and Ara-C. These drugs have both a cytostatic action, arresting cells in DNA synthesis at the time of administration, and a lethal effect, killing S-phase cells; hence they are termed S-phase-specific drugs.

Biochemical mechanisms can account for the cytostatic actions of the drugs. Ara-C is converted to an active form, Ara-C triphosphate, and incorporated into DNA instead of the normal nucleotide, which sterically hinders base stacking [12]. HU inhibits ribonucleoside diphosphate reductase, which catalyses the conversion of ribonucleotides to deoxyribonucleotides [13]. However, the mechanism by which Ara-C and HU cause cell death is still very poorly understood.

The aim of our study has been not only to compare the response of DNA synthesising cells recruited from a quiescent state with those from continually cycling populations, but also to obtain more cytokinetic information regarding different tissue and species sensitivities to the cytostatic and cytotoxic effects of a single dose of each drug.

Methods

Animals. All experiments were performed in male Sprague-Dawley rats weighing 190–250 g or male Balb/c mice weighing 20–25 g.

Cytotoxic drugs. Ara-C (Sigma Chemical Co. Ltd) was dissolved in 0.9% sterile saline and administered IP at either 400 mg/kg or 800 mg/kg body wt. HU (Sigma Chemical Co. Ltd) was dissolved in warm sterile water and administered IP at either 1,500 mg/kg body wt or 3,000 mg/kg body wt. In each experiment, animals received only a single injection of a cytotoxic drug, since we were particularly interested in the long-term fate of the cell population initially affected. For both Ara-C and HU, doses were chosen that were well below the lethal range [14, 19] but have been found to be more than sufficient to produce a consistent amount of intestinal crypt cell

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death [16, 23]. In the case of HU, the dose of 1,500 mg/kg body wt was selected so that these experiments would be directly comparable with a previous study on this topic [10].

Models of stimulated proliferation. The cell kinetic responses of each of the models are well established and have been described in detail in the literature [1–4, 8]. Cytotoxic drugs were administered at or near the time of maximal DNA synthesis in each case. Proliferation of the submandibular and parotid glands was induced by a single IP injection of 83 mg/kg body wt D/L-isoprenaline HCl (IPR) (Sigma Chemical Co. Ltd) dissolved in sterile saline. Drugs were administered 27 h after IPR injection [3, 4].

Accessory sex gland cell proliferation was stimulated 2 weeks after surgical castration by two SC injections of 12.5 mg/kg body wt testosterone (Sigma Chemical Co. Ltd) dissolved in sterile sesame oil, administered 24 h apart. Drugs were administered 48 h after the first injection [1]. Two-thirds partial hepatectomies were carried out by removal of the median and left lateral lobes of the liver under ether anaesthesia. Maximal rates of DNA synthesis occur 24 h after excision in the rat [8] and 36 h after excision in the mouse [2]. Therefore drugs were administered at these respective times.

Preneoplastic foci of rat hepatocytes were induced by the method of Solt and Farber [20]. This procedure produces foci of rapidly proliferating hepatocytes within a 4-week experimental period [21]. Animals received a necrogenic IP dose of 200 mg/kg body wt DEN dissolved in 0.9% sterile saline. After a 2-week recovery period, animals were fed a basal diet containing 0.02% 2-acetylaminofluorene (2AAF) for 1 week. This protocol is thought to selectively inhibit the proliferative activity of normal uninitiated hepatocytes [15]. They were then subjected to two-thirds partial hepatectomy to provide a further hyperplastic stimulus for the initiated cell population. By 48 h after partial hepatectomy a large number of rapidly proliferating basophilic foci were visible. Drug treatment commenced at this time.

Pulse labelling experiments. To allow the time-course of the proliferative response after cytotoxic insult to be followed, a group of animals received single IP doses of Ara-C or HU at the appropriate time after the proliferative stimulus in each model. One control animal (having received the proliferative stimulus but not the drug) and one experimental animal were killed every hour for 24 h after drug treatment. Rats received 0.5 μCi/g body wt tritiated thymidine ([^{3}H]TdR, specific activity 5 Ci/mmol, Amersham International) and mice, 1 μCi/g body wt [^{3}H]TdR 1 h before death. Carnoy's fluid-fixed tissue was dehydrated, wax-embedded, and sectioned at 4 μm onto gelatin-subbed slides. Autoradiographs were prepared by exposure to Kodak AR10 stripping film at 4° C. Mouse tissues were exposed for 2 weeks and rat tissues for 3 weeks. The film was developed in Kodak D19 developer and fixed in Amifix. The sections were lightly stained with Mayer's haematoxylin and 0.1% eosin.

Labelling (I_S), mitotic (I_M), and necrotic (I_N) indices were assessed from counts of 1,000 cells in each tissue and expressed as a percentage. Cells with more than four overlying silver grains were counted as labelled, those from prophase to telophase were counted as mitotic, and those showing pyknosis, karyorrhexis or karyolysis, as necrotic.

Table 1. Rodent tissues studied

Rat and mouse jejunal crypts
Rat and mouse colonic crypts
Rat and mouse oesophagus
Rat and mouse tongue epithelium
Rat and mouse IPR-stimulated parotid and submandibular glands
Rat and mouse testosterone-stimulated seminal vesicles
Rat and mouse regenerating liver
Rat preneoplastic liver foci
Rat 12-day-old liver

Fate of labelled cells experiments. The lethal effects upon DNA synthesising cells at the time of drug administration were followed by pretreating animals with [^{3}H]TdR 30 min prior to drug administration. Three experimental and one control animals were killed 3, 6, and 24 h later. Autoradiographs were prepared, and the percentage of labelled cells showing necrosis (I_{LN}) was assessed from counts of 500 labelled cells.

Results

The rodent tissues studied are summarised in Table 1. In all tissues DNA synthesis was arrested for a period of 4–5 h and mitosis for a period of 3–7 h, and on recovery the proliferative indices (I_S and I_M) exceeded those preceding cytotoxic insult. On the other hand, the susceptibility to drug-induced cell death was markedly different between tissues.

Pulse labelling experiments

Figure 1 illustrates the response of the rat jejunal crypt cells to Ara-C injection, a situation where the majority of S-phase cells were killed by the treatment. The number of labelled cells fell to zero within 1 h of Ara-C injection, and inhibition of DNA synthesis persisted for 5 h. At 6 h the labelling index began to rise again and reached a peak of almost 50% by 14 h. The mitotic index fell to zero by 2 h and remained at this value for 7 h. On recovery, a peak I_M of over 3% was reached by 16 h. By 24 h proliferative indices had almost returned to control values. The time difference between the 50% peak points on the ascending I_S and I_M curves is approximately 7 h, and if the initial repopulation is solely achieved by cells blocked at the G_1 to S barrier, then the value of 7 h should be equivalent to the median $t_S + t_{G2}$ of this early proliferating population. Indeed, a value of 7 h for $t_S + t_{G2}$ in the rat jejunum is in good agreement with fraction of labelled mitoses derived data obtained after HU treatment [23]. Thus it seems unlikely that surviving S-phase cells contribute to the repopulation of the jejunal crypts to any significant extent. The necrotic index in this tissue begins to rise 1 h after Ara-C treatment, and by 5 h reaches a peak of approximately 30%, comparable to the normal labelling index in control animals. This implies that nearly all cells in S-phase at the time of cytotoxic drug administration were killed. The necrotic index then falls rapidly, very few necrotic cells being observed after 10 h and none at all by 18 h.

In contrast, the response of the mouse oesophagus (Fig. 2) represents a situation where cell death is not caused by the treatment. DNA synthesis was totally inhibited within 1 h, and the number of mitotic cells reduced to zero within 2 h. This time difference probably represents cells already in G_2 and

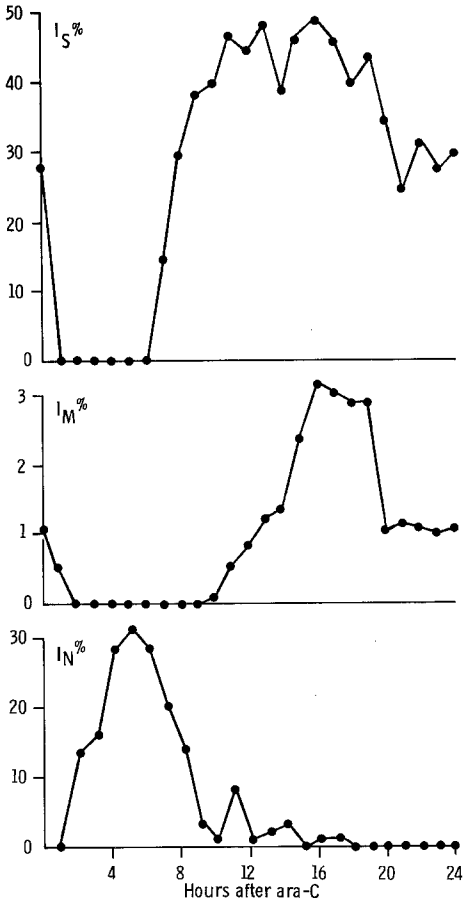

Fig. 1. Response of rat jejunal crypts to 400 mg/kg Ara-C

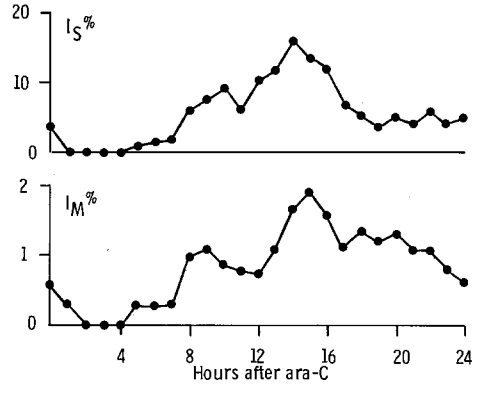

Fig. 2. Response of mouse oesophagus to 400 mg/kg Ara-C

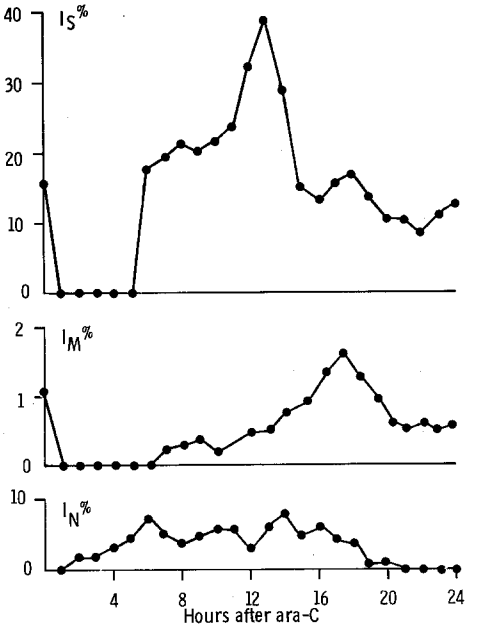

Fig. 3. Response of testosterone-stimulated seminal vesicles in the castrated mouse to 400 mg/kg Ara-C

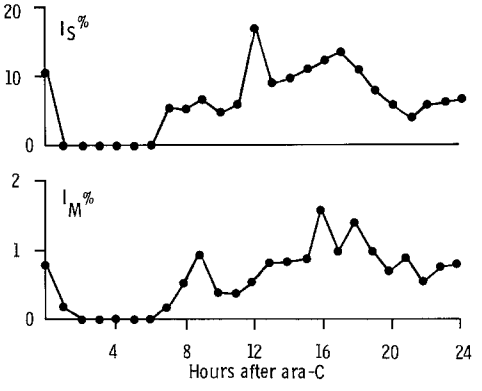

Fig. 4. Response of IPR-stimulated acinar cells from mouse submandibular gland to 400 mg/kg Ara-C

mitosis at the time of Ara-C injection, completing mitosis. Both indices (I_S and I_M) begin to rise concomitantly at 4 h, which is consistent with arrested cells continuing their progress through the cycle upon recovery. I_S reaches a peak of 14% by 14 h and I_M a peak of nearly 2% by 15 h; both have almost returned to control values by 24 h.

By comparison, Fig. 3 demonstrates an intermediate situation in which some S-phase cells are killed by the treatment whilst others survive and resume DNA synthesis at a later time. Necrosis is apparent by 2 h after Ara-C injection; I_N rises to values of approximately 8% by 6 h but by 22 h no necrotic cells remain.

Figures 4 and 5 illustrate the response of two other tissues to Ara-C, namely the IPR-stimulated mouse submandibular gland and the rat tongue epithelium. In neither case was any cell death evident, and mitotic activity resumed more or less concomitantly with DNA synthesis. In the tongue epithelium (Fig. 5) peak values of I_S and I_M on recovery were almost six-fold greater than control values, suggesting a higher degree of cell synchronisation than seen in the other treated tissues. However, surprisingly, only a low level of mitotic activity was observed between 5 and 11 h after Ara-C injection, which may indicate a variable $t_S + t_{G2}$ in the early proliferating population.

In none of the models of liver growth was necrosis observed at any time after drug treatment. In each case, DNA synthesis was inhibited within 1 h of injection and there was an overshoot in I_S compared with control values upon recovery (Fig. 6).

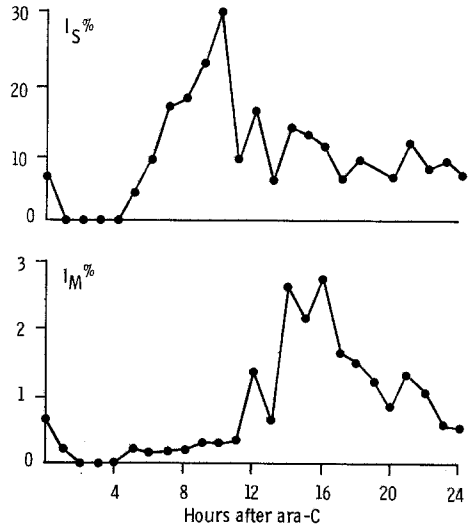

Fig. 5. Response of basal layer of rat tongue epithelium to 400 mg/kg Ara-C

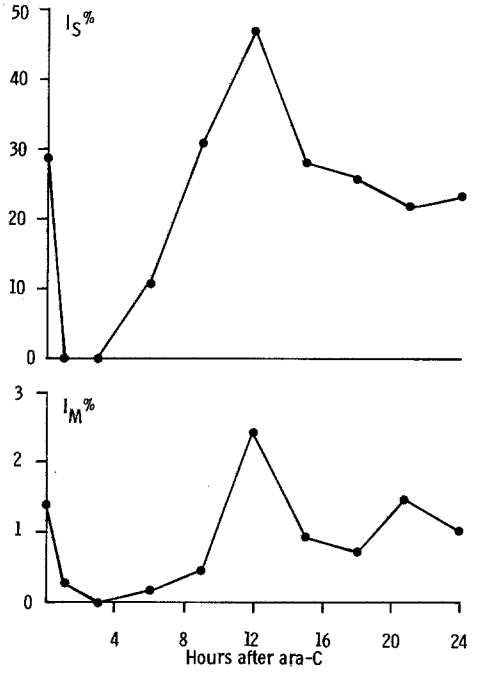

Fig. 6. Response of DEN-induced basophilic foci in rat liver to 400 mg/kg Ara-C. No proliferation was seen in the surrounding apparently normal tissue

Fate of labelled cells experiments

It has been demonstrated that the [^{3}H]TdR flash-labelling index is a good estimate of the fraction of cells in DNA synthesis in both the normal mouse colon [11] and the regenerating rat liver [7]. Hence in the present experiments the thymidine labelling index was considered to be a reliable assessment of the cells in DNA synthesis, and thus a measure of the target population of HU and Ara-C.

Table 2 illustrates the effect of HU and Ara-C on various DNA-synthesising cell populations in the rat, the cells having been prelabelled with [^{3}H]TdR 30 min before cytotoxic drug treatment. In the small intestine both drugs brought about the rapid death of the majority of labelled cells, and at 6 h after injection about 90% of the labelled cells were found to be necrotic. In the large intestine the drugs were less effective and at 6 h after injection only about 25% of the labelled cells were necrotic. In both these regions of the gut all the necrotic cells were removed within 24 h of injection. On the other hand, two other continually renewing populations in the rat, the oesophagus and tongue epithelium, showed no evidence of necrosis (see Table 4). Amongst the stimulated populations in the rat the most susceptible was the seminal vesicle, with approximately 25% of the labelled cells appearing necrotic 6 h after drug treatment. A very low level of cell death was seen in the parotid gland, but no cell death was seen in the submandibular gland, regenerating liver, preneoplastic liver cell foci, or postnatal liver. The results from these experiments are in broad agreement with the pulse labelling experiments. Only in the jejunum where most S-phase cells became necrotic could a clear temporal separation in the resumption of I_S and I_M be discerned (Fig. 1).

Table 3 shows the results of similar experiments performed in the mouse. As in the rat, most S-phase cells in the mouse jejunal crypts succumbed to the lethal effects of the drugs, but in the mouse colonic crypts about 50% of labelled cells were necrotic 6 h after drug treatment, as against only 25% in the rat.The response of stimulated mouse populations susceptible to drug treatment was comparable to those of the rat, once again, only the seminal vesicles showing appreciable degrees of necrosis. The mouse oesophagus, IPR-stimulated submandibular gland, and regenerating liver showed no necrosis. Table 4 summarises the results of all experiments.

Discussion

Our studies show populations resistant and susceptible to the lethal actions of HU and Ara-C both in continually renewing systems and in cell populations stimulated to divide from a quiescent state. They therefore do not support the hypothesis of Farber and Baserga [10], who suggested the former to be susceptible to the drugs and the latter resistant due to a biochemical difference between G_1 and G_0 cells. Their studies were based on three models of stimulated proliferation; the regenerating liver, the folic acid-stimulated kidney, and the IPR-stimulated parotid gland. The dose of HU used in their studies was 1,500 mg/kg, which is consistent with our experiments, but levels of necrosis were largely assessed at only one time point, 3 h after drug administration. Our results show the peak time of expression of necrosis to be later than this.

It is unlikely that the former G_0 state of hepatocytes is the factor determining the resistance to drug-induced cell death in these experiments. We have found that drug-induced cell death cannot be observed in hepatocytes in the immediate postnatal period where cells are proliferating during developmental growth. We looked at the response at this time because this represents a model of hepatocyte proliferation where cells have not recently been induced to divide from a quiescent state [18]. It therefore appears that an inherent metabolic property of the liver accounts for the complete resistance to the cytocidal effects of the drugs, rather than a resistance directly associated with the former proliferative state.

Carcinogen-induced drug resistance in rat hepatocytes has been a subject of wide interest [5], but the complete resistance of DEN-induced basophilic foci to the lethal effects of HU and Ara-C has not been previously demonstrated. These foci have

Table 2. Percentage of labelled cells showing necrosis (I_{LN}) in rat tissues[a]

Tissue	Hours after drug	$I_{LN} \pm SD$			
		HU (mg/kg body wt)		Ara-C (mg/kg body wt)	
		1,500	3,000	400	800
Jejunal crypts of small intestine	3	73.6 ± 5.0	74.7± 9.7	76.8 ± 4.9	80.5 ± 6.2
	6	92.0 ± 3.6	91.8 ± 3.6	88.1 ± 5.6	92.1 ± 2.6
	24	0	0	0	0
Colonic crypts	3	9.8 ± 1.3	11.6 ± 2.7	9.5 ± 1.3	11.6 ± 2.5
	6	26.9 ± 4.7	26.2 ± 5.1	25.3 ± 3.9	28.4 ± 2.3
	24	0	0	0	0
IPR-stimulated parotid gland	3	0	0	0	0
	6	0	0	0	0
	24	0.6 ± 0.25	1.3 ± 0.6	0	1.2 ± 0.3
Testosterone-stimulated seminal vesicles in the castrated animal	3	2.7 ± 0.9	2.3 ± 0.6	6.0 ± 1.5	6.3 ± 0.75
	6	13.8 ± 2.8	19.5 ± 4.9	24.5 ± 4.9	25.7 ± 2.2
	24	0	0	0	1.1 ± 0.95

[a] I_{LN} in control tissues was < 0.3%

Table 3. Percentage of labelled cells showing necrosis (I_{LN}) in mouse tissues[a]

Tissue	Hours after drug	$I_{LN} \pm SD$			
		HU (mg/kg body wt)		Ara-C (mg/kg body wt)	
		1,500	3,000	400	800
Jejunal crypts of small intestine	3	81.7 ± 2.1	91.8 ± 2.4	88.2 ± 3.3	89.3 ± 3.2
	6	91.5 ± 6.1	91.9 ± 2.2	94.5 ± 3.1	96.1 ± 2.8
	24	0	0	0	0
Colonic crypts	3	12.1 ± 1.1	12.7 ± 1.4	13.0 ± 2.5	13.4 ± 1.9
	6	50.3 ± 8.2	47.7 ± 1.9	52.5 ± 3.8	53.7 ± 2.6
	24	0	0	0	0
Basal layer of tongue	3	0	0	0	0
	6	0	1.2 ± 0.3	0	0.83 ± 0.55
	24	0	0	0	0
IPR-stimulated parotid gland	3	0	0	0	0
	6	0	0	0	0
	24	5.6± 3.7	5.8 ± 2.1	5.9 ± 1.9	5.4 ± 1.1
Testosterone-stimulated seminal vesicles in the castrated animal	3	5.0 ± 1.6	4.9 ± 1.9	6.1 ± 2.1	6.0 ± 2.9
	6	13.8 ± 3.0	15.8 ± 3.2	13.6 ± 2.3	14.5 ± 2.3
	24	0	0	0	0

[a] I_{LN} in control tissues was < 0.2%

Table 4. Summary of results of all experiments in the study

Tissues totally resistant to the lethal actions of HU and Ara-C	Tissues showing varying degrees of necrosis
Rat and mouse oesophagus	Rat and mouse jejunal crypts
Rat and mouse IPR-stimulated submandibular gland	Rat and mouse colonic crypts
Rat and mouse regenerating liver	Rat and mouse IPR-stimulated parotid gland
Rat liver preneoplastic foci 48 h after partial hepatectomy	Rat and mouse testosterone-stimulated seminal vesicles
Rat tongue epithelium	Mouse tongue epithelium
Rat 12-day-old liver	

many altered enzymic activities [9]. However, their response to the drugs is similar to that of normal hyperplastic liver during regeneration. DNA-synthesising cells from different tissues show very variable sensitivities to drug lethality in these studies, although adequate drug concentrations were present in all tissues to completely arrest DNA synthesis. The variable cytotoxic effects are likely to be due to metabolic enzyme levels and tissue distribution of the drugs. However, there is no doubt that sensitivity to drug-induced cell death is not unique to continual renewal populations as previously suggested.

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