Europ. J. Cancer, Vol. 16, pp. 663–669

0014-2964/80/0501-0663 $02.00/0

Cell Proliferation and Ageing in Mouse Colon. II. Late Effects of Repeated X-Irradiation in Young and Old Mice

E. HAMILTON* and L. M. FRANKS

Department of Cellular Pathology, Imperial Cancer Research Fund, Lincoln's Inn Fields, London, United Kingdom

Abstract—*Cell kinetic parameters in the descending colon of unirradiated mice,* 3–30-months-*old were compared with those in mice irradiated repeatedly from the age of* 6 *or* 24 months. *The latter animals were given* 1250 rad *local X-irradiation to the colon every* 6 weeks. *Dose–survival curves showed the colon crypts of* 6 *and* 24-months-*old mice were similarly radiosensitive.*

In unirradiated mice the number of crypts per colon section decreased significantly at 30 months, *but no significant age-related changes were seen in crypt size or labelling index (LI). Cell proliferation returned to control levels within* 6 weeks *of each X-ray dose and remained at this level for* 20 weeks *after the final dose. Later, cell proliferation in the irradiated colon fell significantly below control. A total of* 6 *or* 7 *doses each of* 1250 rad *produced only* 1 *colon carcinoma amongst* 50 *mice kept until they died.*

INTRODUCTION

AGE-RELATED changes in cell proliferation have been reported in several regions of the mouse gut. In mouse small intestine the length of the DNA synthesis (S) phase and the total cell cycle length increase gradually up to 34 months of age [1]. The length of the G_1 phase is longer in the colon cells of 21-month-old than in 12-month-old mice [2]. This gives rise to a lower labelling index (LI) in the older mice.

Age-related changes in gut cell proliferation might be related to the number of divisions the crypt progenitor cells undergo. In tissue culture, normal mouse cells go through only 8–10 population doublings before they transform or die [3]. The aim of the present work was to see if mouse gut cells *in vivo* have a similar 'proliferative limit' at which they become neoplastic or cease dividing. To test this the descending colon was repeatedly depopulated, by local X-irradiation. Extra population doublings were required to repopulate the epithelium from the cells surviving each irradiation. This procedure might produce tumours by direct radiation carcinogenesis, rather than by transformation of cells at a 'proliferative limit'. Single-dose local X-irradiation produced tumours in the colon and rectum of both rats and mice [4–6].

Tumour incidence increased with radiation dose and up to 95% of the animals developed tumours within 20 months of irradiation [4].

In the present paper, the effects on tumour incidence and cell proliferation of repeated colon X-irradiation at 6-week intervals is described. This is compared with cell proliferation in the colon of untreated mice, 3–30-months-old.

MATERIALS AND METHODS

Male C57BL/Icrf a^t mice, aged under standard animal house conditions [7] were used. The mice were restrained in a special jig [8], and the colon was exposed to 220 KVp X-rays through a 2.5 × 2 cm hole in a lead shield which covered the body. The animals breathed 95% O_2, 5% CO_2 during the irradiations, to increase the radiosensitivity of the colon.

Accepted 8 October 1979.
*Present address: Department of Oncology, Middlesex Hospital Medical School, London W1P 7PN, U.K.

The microcolony assay [9] was used to construct crypt dose–survival curves. Groups of 4–6 mice were irradiated at each dose and killed $4\frac{1}{2}$ days (4-month-old mice) or $5\frac{1}{2}$ days (24-month-old mice) later. The surviving crypts were counted in H and E stained sections from the irradiated area of colon of each mouse. Crypts were counted in unirradiated mice of the same age, for the calculation of proportional survival.

One hundred and five 6-month-old animals were given 1250 rad X-rays to the colon at 6-week intervals. Groups of 5 mice were killed at intervals after each dose. Six weeks after the 6th dose half the remaining animals were given a 7th dose and both groups were kept alive for as long as they were healthy. Twenty-five sham-irradiated control mice were killed, in groups of five, throughout the experiment. Twenty-five 24-month-old (old) mice were given four doses of 1250 rad X-rays, at 6-week intervals. Four sham-irradiated controls were killed with the last group of irradiated animals.

All irradiated or untreated mice were killed between 14.30 and 16.00 hr. Each was injected with 30 μCi ^{3}H-thymidine (spec. act. 5 Ci/mM) 40 min before killing. Sections and autoradiographs were prepared and counted as described previously [8, 10].

RESULTS

C57BL/Icrf a^t virgin male mice live for a maximum of 36 months, with 50% of the population dying within the first 28 months [7]. Figure 1A shows the average number of crypts in transverse sections of the descending colon, cut perpendicular to the gut lumen, from groups of 4–5 untreated mice, 4–30-months-old. There were significantly fewer crypts per section in 30-month-old mice than in those 4–18-months-old. In Fig. 1B the average number of cells per U-shaped, longitudinal crypt section (cut parallel to crypt lumen) in mice of different ages is shown. There was no clear age-related change. The percentage of labelled cells (LI) in the colon epithelium of different aged mice is plotted in Fig. 2A. The differences were not significant, but the LI was lowest at 18 months. Figure 2B shows the total number of labelled cells per 5 μm circular colon section, calculated from:

$$\text{LI} \times \text{cells/crypt} \times \text{crypts/section}.$$

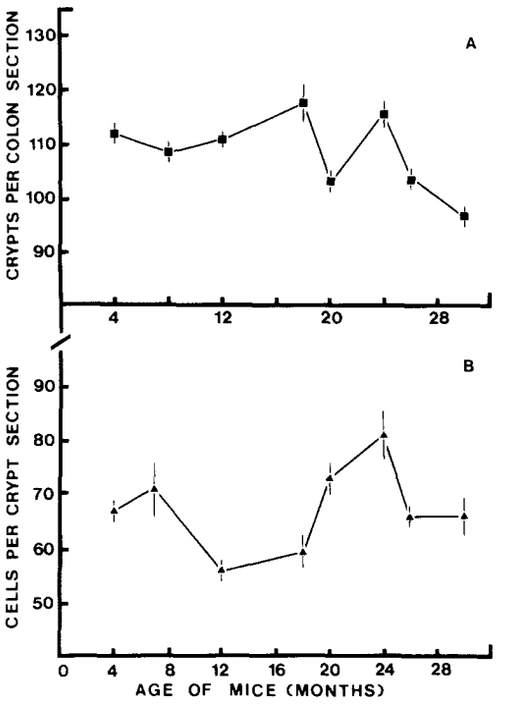

Fig. 1. (A) Number of crypts per 5 μm transverse section, cut perpendicular to the gut lumen, of descending colon in mice 4–30-months-old. Mean and standard error from groups of four or five mice are plotted.

(B) Mean number of cells per axially sectioned colon crypt in mice of different ages. Counts included both sides of the crypt, from base to surface epithelium.

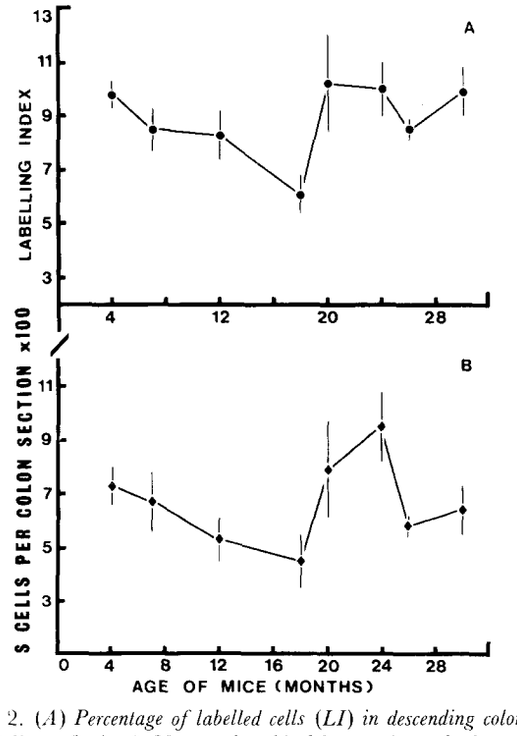

Fig. 2. (A) Percentage of labelled cells (LI) in descending colon epithelium of mice 4–30-months-old. Mean and standard error from groups of four or five mice are plotted.

(B) Number of DNA synthesising (S) cells per circular colon section in mice of different ages. Calculated from: LI × cells per crypt × crypts per section.

This was highest in 20 and 24-month-old mice, but the standard errors were large, so the differences were not significant.

Irradiated mice

Single dose survival curves for descending colon crypts in 4 or 24-month-old mice are shown in Fig. 3. The parameters of the surviving curve for 4–5-month-old mice are: $D_0 = 299.5 \pm 14.5$ rad and extrapolation number (N) $= 25.0 \pm 5.1$. For 24-month-old mice, $D_0 = 275.9 \pm 14.6$ rad and $N = 20.3 \pm 5.9$. The two sets of data are, therefore, not significantly different.

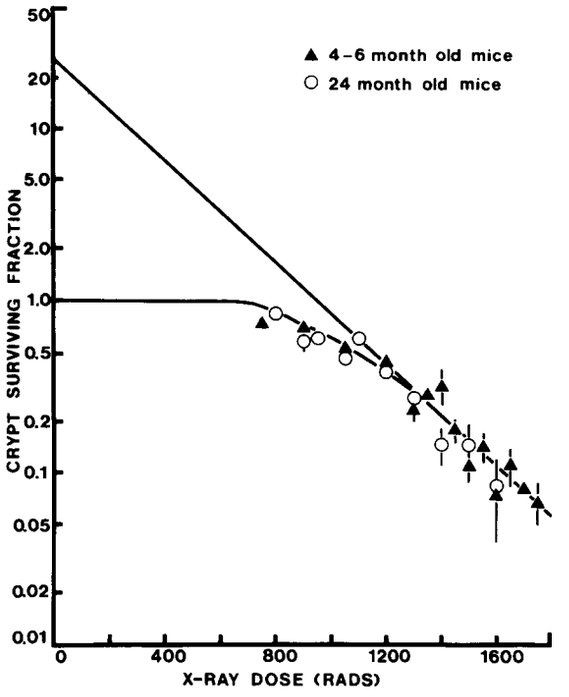

Fig. 3. X-ray survival curves for descending colon crypts in mice of two ages. Mice were given local irradiation to the colon and microcolonies were counted 4–5 days *later. Mean and standard error from groups of four to six mice are plotted.*

Very few mice died from acute effects after the irradiation of a 2 cm length of gut. Over a 3-yr period 170 4–6-month-old mice were given a single dose of 1250 rad X-rays to the descending colon and were kept alive for more than 5 days (see also [10]). One mouse died 19 days after irradiation and all the rest survived at least 30 days. Of 74 24-month-old mice given a single dose of 1250 rad, one mouse died 6 days after irradiation and seven died on day 11 or 12.

In the 24-month-old mice given repeated irradiation no changes other than those associated with repopulation after X-irradiation and with normal ageing were seen in the colon [7]. However, all these mice were killed within 7 weeks of irradiation.

Of the 105 6-month-old mice irradiated repeatedly, two were found dead within 10 days of the 6th dose. A total of six mice died as a result of obstruction in the irradiated colon and rectum, 16 or more weeks after the sixth and seventh doses. In these animals the outer muscle coat of the gut had degenerated in many places and in some mice this had led to the formation of macroscopic nodules over the outside of the colon. The crypts were very hyperplastic but the surface epithelium of the gut had degenerated. In 10 of the mice the bases of some crypts in the irradiated area were trapped below the muscularis mucosae. However, they did not penetrate the outer gut muscle and no abnormal cells were seen. In the colon of only one mouse, killed 16 weeks after the 6th dose, was there evidence of neoplasia. In this animal there was focal necrosis in the surface epithelium, with bleeding into the gut lumen. Below these necrotic areas the stroma was bare of crypts. In other areas of the colon there were hyperplastic crypts with a very high LI and the crypt bases had penetrated the muscularis mucosae. This was associated with nests of cytologically abnormal epithelial cells in the lamina propria. This morphology suggested part of the epithelium had undergone a malignant change, but the cells were not highly invasive.

Fifty mice were kept alive for more than 6 weeks after the sixth and seventh doses. Only one of these animals showed evidence of colonic neoplasia and 10 had crypts 'trapped' below the muscularis. No young irradiated mice showed abnormalities in the colon other than those described above.

Table 1 shows the proportion of crypts per section surviving in the colon of irradiated mice, 1 and 3–7 weeks after each irradiation. There was an increase in the number of surviving crypts after each dose. In 24-month-old mice the proportional increase was 0.28, relative to control, after the first dose, but only 0.02 after the fourth dose. In the 6-month-old mice, there was no evidence of a successively smaller increase in crypt numbers after each dose. In no case did the number of crypts per section return to that in control mice within 7 weeks of irradiation (Table 1). Figure 4 shows that it took 25–30 weeks for crypt numbers to return to control values after six and seven doses of X-rays.

An estimate can be made of the proportion of crypts killed by each irradiation, from the

Table 1. *Proportional crypt survival after one to seven doses of* 1250 rad *X-rays, calculated from the numbers of crypts in unirradiated age-matched controls*

		Mice 6-months-old at start			Mice 24-months-old at start		
Dose	Weeks	Kill*	Surviving crypts	Increase†	Kill*	Surviving crypts	Increase†
		0.43			0.61		
1	1		0.57 ± 0.03	0.14		0.39 ± 0.04	
	4		0.71 ± 0.09				0.28
	6					0.67 ± 0.03	
		0.40			0.26		
2	1		0.31 ± 0.03	0.29		0.41 ± 0.04	
	3		0.60 ± 0.03				0.15
	5					0.56 ± 0.05	
		0.14			0.28		
3	1		0.46 ± 0.04	0.11		0.28 ± 0.03	
	4		0.57 ± 0.04				0.13
	6					0.41 ± 0.04	
		0.22			0.06		
4	1		0.35 ± 0.05	0.14		0.35 ± 0.03	0.02
	7		0.49 ± 0.04			0.37 ± 0.03	
		0.10					
5	1		0.39 ± 0.02	0.07			
	7		0.46 ± 0.02				
		0.08					
6	1		0.38 ± 0.03	0.24			
	7		0.62 ± 0.03				
		0.25					
7	1		0.37 ± 0.04	0.11			
	7		0.48 ± 0.04				

*Crypts killed by each irradiation, calculated from final measurement after the previous dose.
†Increase in crypts between first and last measurements after each dose.

last measurement after the previous dose. As Table 1 shows the crypts killed by each successive dose fell, in both young and old mice, although there appears to be an increase in crypt killing by the seventh dose. This decrease in the effectiveness of each X-ray dose for crypt killing has been investigated in greater detail [11].

The repeatedly irradiated mice were kept alive for as long as possible, in order to measure the incidence of colon tumours. Of the mice given six and seven doses, 60% had to be killed when the skin over the entire irradiated area become necrotic. This necrosis developed much earlier in cages where the mice fought when young. Colon proliferation parameters were, therefore, measured in mice killed at random intervals after irradiation.

Table 2 shows the level of colon cell proliferation in mice killed 6–7 weeks after irradiation, expressed as a percentage of control values. In all these groups of mice there were

Table 2. *Proliferation parameters, expressed as percentage of control, in irradiated colon of mice killed* 6–7 weeks *after last dose of* 1250 rad

Doses	Age at start (months)	Age at death (months)	No. of mice	LI	Cells per crypt	S cells per circular colon section
1	24	26	3	117	114	110
2	24	27	6	124	115	97
3	24	29	1	105	141*	96
4	24	30	4	132	149*	122
4	6	12	1	72	136*	64
5	6	13	1	144*	128*	130
6	6	15	6	165	129*	169
7	6	17	6	143	108	103

*Significantly different from control ($P < 0.05$).

fewer than the control number of crypts per section (Table 1). However, these crypts were larger and mostly had a higher LI than the control (Table 2). The number of S cells per colon section was, therefore, not significantly different from the control (the latter measurement, since it is derived from several others, has quite large errors).

Table 3 shows proliferation parameters in the colon of young mice killed at longer intervals after six or seven doses. The number of crypts per section returned to normal within 31 and 25 weeks of six and seven doses respectively (Fig. 4). Up to 20 weeks after the last dose the number of S cells per colon section was rather above the control, due to an increased LI and crypt size (Table 3).

However, when the crypt numbers returned to control levels the LI fell, as did crypt size in some animals. This resulted in lower numbers of S cells per colon section, in some cases significantly below controls (Table 3).

DISCUSSION

Work with the small intestine indicates that cell kinetic changes become marked as mice approach old age (30 months or more) [1]. Figures 1 and 2, however, show only one significant age-related change—there are fewer crypts per colon section in 30-month-old mice than in younger animals (Fig. 1A). In the descending colon of young C57B1 a^t mice there is a large diurnal variation in both LI and the number of cells per crypt [12]. The peak values for both these parameters occur at midday. None of the mice in the groups shown in Figs. 1 and 2 had values for colon LI

Table 3. Young mice killed after six or seven doses of 1250 rad. *Colon proliferation parameters expressed as percentage of age-matched controls*

Doses	Weeks	Age at death (months)	No. of mice	LI	Cells per crypt	S cells per circular colon section
6	7	15	6	165	129*	169
	13	17	7	174*	117	121
	29	21	2	66	155	59
	31	21	2	82	88	74
	49	26	3	91	119	94
7	7	17	6	143	108	103
	15	19	3	110	114*	90
	20	20	2	112	161*	121
	23	21	1	70	119*	71
	25	21	2	63	73*	38*
	43	26	1	83*	103	62*

*Significantly different from control ($P<0.05$).

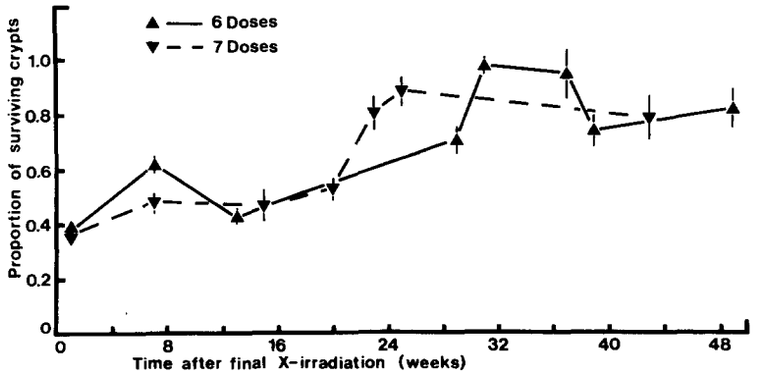

Fig. 4. Late changes in the crypts per colon section, calculated as a proportion of the number in age-matched controls, in 6-month-*old mice given six or seven doses of* 1250 rad *X-rays at* 6-week *intervals.*

or cells per crypt outside the range seen in young mice between 08.00 and 16.00 hr [12]. The data in Figs. 1B and 2 suggest, therefore, that the timing of the peaks in diurnal variation may become more variable as the animals age. It is impossible, however, to tell whether the level of cell proliferation in the colon changes with age.

The data in Fig. 3 and the similar values for D_0 and N derived from them suggest that the crypts of 4 and 24-month-old mice contain similar numbers of cryptogenic cells, of a similar radiosensitivity. The 2-yr-old animals, however, are more liable to die from the acute effects of gut X-irradiation. A dose of 1250 rad to the colon left 0.4 of the crypts surviving in old mice (Table 1) and eight of 74 mice died within 12 days of the irradiation. In young mice, when crypt survival was reduced to the same level (Table 1), only two of over 200 mice died. Repopulation of the irradiated colon is slower in old than in young mice [10], and this may contribute to the early deaths. Old mice may also be more susceptible to humoral changes occurring after irradiation as they tend to die sooner after whole-body irradiation than do young mice (unpublished data).

The incidence of colon tumours in young mice after total doses of 7500 and 8750 rad was extremely low. This is in marked contrast to other workers [4–6] who found large numbers of tumours after single doses or 2–3 weekly fractions of X-rays to the colon or rectum. However, the present work differs from the previous reports in several respects. Single doses of 1250 rad are much lower than the doses used previously (1600–6500 rad), where tumour incidence increased with the size of dose. This suggests, firstly, that the tumour-producing lesion can be repaired in the 6 weeks between repeated irradiations. Secondly, the level of tumour production is inversely related to the level of crypt survival. A smaller number of surviving cells would have to undergo more rapid divisions in order to repopulate the irradiated gut within 2–3 weeks [4, 10]. These rapid divisions might allow the expression, rather than repair, of a neoplastic lesion.

In addition, the previous workers used animals no older than 3 months—younger than those in the present study. In the small intestine, the cryptogenic cell population of young mice contains a larger percentage of dividing cells, cycling more rapidly than in adult animals [13]. This may also be the case in the colon, where compensatory proliferation occurs more slowly in old animals [10]. Fewer cycling cryptogenic cells at the time of irradiation might also allow for repair of a neoplastic lesion.

Increasing the number of divisions colon epithelial cells undergo neither produces tumours nor causes proliferation to cease. Repopulation of a crypt from one surviving cell to the 350 or so found normally [12] requires eight extra divisions. This number of extra divisions will not have been required after each dose, as successive treatments killed fewer crypts (Table 1). However, seven doses may have increased the total number of divisions by about 30. This is far in excess of the population doublings mouse cells undergo *in vitro* before they transform or die [3]. The fact that the colon epithelium of 2-yr-old mice was able to repopulate itself four times (Tables 1 and 2) suggests that any 'proliferative limit' is far in excess of the number of divisions the tissue normally undergoes.

However, a proliferative limit, or defect, may become apparent many months after a course of repeated irradiations. At intervals of more than 20 weeks after the last dose, the normal number of S cells per colon section was not maintained, due to a fall in the LI (Table 3). This may be because of a limit on the crypt cell division potential. It is also possible that the stroma and blood vessels below the epithelium express late radiation damage, and proliferation is restricted by lack of nutrients and oxygen. However, although thick-walled blood vessels were seen in the irradiated area, these also occur in the colon of untreated old mice. There was also no evidence of a reduction in the amount of ^{3}H-thymidine reaching the irradiated colon epithelium. However, the possibility of late vascular changes affecting the proliferation rate cannot be ruled out. The skin necrosis, for which many animals were killed, was probably caused by late radiation effects on the dermis.

The colon cell kinetic responses to repeated irradiation were complex and they suggest proliferation is regulated at two levels. Firstly, there is a control on the number of crypts per section. This causes the number of crypts to rise slowly to the control level (Table 1, Fig. 4) but then allows no further increase in numbers. This mechanism may either cease to function in 2-yr-old mice after several doses, or it may act more slowly after each irradiation in these animals (Table 1). Secondly, there is a mechanism which controls the number of S cells per colon section. This 'makes

up' for the deficiency in number of crypts, by increasing their size and LI. This is a rapidly acting mechanism, bringing S cells per section back to normal within 2 weeks of irradiation in young and old mice [10]. It does not cease to operate after several doses in 2-yr-old mice (Table 2), but when the number of crypts has returned to normal, the number of S cells per section falls (Table 3). This might be due to an 'overcompensation' in the control of S cells per section, once the need to maintain elevated levels of proliferation per crypt is over.

In summary, these data do not answer the question of whether a proliferative limit exists in the gut, but they suggest that any such limit lies far beyond the number of divisions cells normally undergo in their lifetime. Increasing the number of cell population doublings in the gut does not appear to increase its susceptibility to cancer induction.

REFERENCES

1. S. Lesher and G. A. Sacher, Effects of age on cell proliferation in mouse duodenal crypts. *Exp. Geront.* **3,** 211 (1968).
2. J. D. Thrasher, Age and the cell cycle of the mouse colonic epithelium. *Anat. Rec.* **157,** 621 (1967).
3. G. J. Todaro and H. Green, Quantitative studies of the growth of mouse embryo cells in culture and their development into established lines. *J. Cell Biol.* **17,** 229 (1963).
4. F. Hirose, K. Fukazawa, H. Watanabe, Y. Terada, I. Fujii and S. Otsuka, Induction of rectal carcinoma in mice by local X-irradiation. *Gann* **68,** 669 (1977).
5. A. Y. Rostom, S. I. Kauffman and G. G. Steel, Influence of misonidazole on the incidence of radiation-induced intestinal tumours in mice. *Brit. J. Cancer* **38,** 530 (1978).
6. D. L. Denman, F. R. Kirchner and J. W. Osborne, Induction of colonic adenocarcinoma in the rat by X-irradiation. *Cancer Res.* **38,** 1899 (1978).
7. C. Rowlatt, F. C. Chesterman and M. U. Sheriff, Lifespan, age changes and tumour incidence in an ageing C57B1 mouse colony. *Lab. Animals* **10,** 418 (1976).
8. E. Hamilton, Differences in survival of mouse colon crypts after whole- or partial-body irradiation. *Int. J. Radiat. Biol.* **31,** 341 (1977).
9. H. R. Withers and M. M. Elkind, Microcolony survival assay for cells of mouse intestinal mucosa exposed to radiation. *Int. J. Radiat. Biol.* **17,** 261 (1970).
10. E. Hamilton, Cell proliferation and ageing in mouse colon. I. Repopulation after repeated X-ray injury in young and old mice. *Cell Tiss. Kinet.* **11,** 423 (1978).
11. E. Hamilton, Induction of radio-resistance in mouse colon crypts by X-rays. *Int. J. Radiat. Biol.* **36,** 537 (1979).
12. E. Hamilton, Diurnal variations in proliferative compartments and their relation to cryptogenic cells in the mouse colon. *Cell Tiss. Kinet.* **12,** 91 (1979).
13. W. R. Hanson, R. J. M. Fry and A. R. Sallese, Cytotoxic effects of colcemid or high specific activity tritiated thymidine on clonogenic cell survival in $B6CF_1$ mice. *Cell Tiss. Kinet.* **12,** 569 (1979).