The behaviour of proliferative intestinal epithelium cells may be regarded as an excellent way of evaluating the effect of ionizing radiation on tissues with high proliferative activity.

Obviously only partial information on the localization of S-phase cells in the proliferative compartment can be gained from quantitative morphologic parameters (total number of cells, labelling and mitotic indices).

Previous investigations on the small intestine indicated a different distribution of S-phase cells in the crypts of control rats (CAIRNIE et coll. 1965, CAIRNIE 1967, CHENG & LEBLOND 1974a, BECCIO et coll. 1983, AL-DEWACHI et coll. 1976). Moreover they showed that the cells took up 3H Thymidine with varying frequency according to the time of day when the animals were killed (BECCIO et coll. 1983).

The existence of circadian phenomena also in the functional activity of the small intestine (SAITO et coll. 1975, STEVENSON & FIERSTEIN 1976, BECCIO et coll. 1977) led to an investigation of the conditions when tissues may respond in different ways to the same lesion-inducing treatment. The modifications of the S-phase cell distribution in the crypts during acute radiation injury in animals exposed at different times of the day are now reported.

Invertase activity, a brush border enzyme synthesized by epithelial cells during and after the differentiation process (NORDSTRÖM et coll. 1968, JAMES et coll. 1971, ALPERS 1977) was also evaluated.

Material and Methods

Ninety-six 10- to 12-week-old female Wistar rats weighing between 180 and 200 g were used. They were kept for 2 to 3 weeks at a constant light-darkness cycle (6.30 a.m. to 6.30 p.m.), with water and food ad libitum. Cage conditions were strictly controlled.

Anesthetized animals were exposed on the whole abdomen (field 5 cm x 5 cm) to a γ-ray source (telecobalt unit). Four different groups were irradiated with a dose of 8 Gy at midnight (group A), at 6.00 a.m. (group B), at noon (group C), and at 6.00 p.m. (group D).

The rats were killed in groups of 3 at 6, 12, 20, 36, 48 and 72 h after irradiation. Control animals, 6 in each group, were killed at the same time of the day. One hour before killing, 3.7 MBq (100 μCi) of 3H Thymidine, specific activity 74 GBq/mmol (2 Ci/mmol), were injected intraperitoneally.

Immediately after killing, the small intestine was cut longitudinally and washed in cold saline. Then, one cm segment was cut about 15 to 20 cm from the pylorus and spread out on a pasteboard piece.

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specimen was fixed in Carnoy's solution and embedded in paraffin. Sections of 5 μm were cut with particular care to obtain well-aligned crypt-villus systems.

An Ilford K5 emulsion was used for autoradiography.

Fifty left sides of Lieberkuhn crypts were counted for each control animal, while 40 formations were counted in irradiated animals because of the difficulty in finding well-aligned formations in the injured epithelium. Other conditions concerning the processing of the data by a computer have been reported previously (BECCIOLINI et coll. 1983).

In the animals killed at 48 and 72 h, when epithelium alterations were at their greatest, it was possible to count only in less injured areas, where some kind of alignment was present.

In a few groups, only one or two animals showed a number of crypt-villus formations which were high enough for a significant number of counts. In these cases, the mean curve without ±SE limit values is reported; results have shown that this curve is generally representative of the whole group.

The position of mitotic cells was also recorded at first, but their reduced frequency and the fact that they varied greatly in the different animals did not allow a sufficiently accurate determination of this parameter.

In these and other animals treated in the same way, but not injected with 3H Thymidine, the small intestine was divided into 5 tracts which were washed, weighed and homogenized with water 10 per cent (w/v). After 900 g centrifugation the supernatant was used for invertase activity assay (DAHLQUIST 1964). The activity was expressed as unit/g of protein where 1 unit is the activity that can hydrolyze 1 μmol of substrate per minute. The protein content was determined according to LOWRY et coll. (1951).
**Results**

Morphologic observations have been reported previously (Becciolini et coll. 1982). The results can be summarized as follows: at early intervals after exposure radiation injury was limited to the proliferative compartment. Subsequently, the injury became progressively worse; the epithelium appeared reduced in height, the crypts consisted of a few cells altered in shape and size. At 48 h the injury affected the villi also, which were collapsed, conglutinated and covered by a thin epithelium with vacuolated cells at the top.

Recovery set in in the crypts 72 h after irradiation but in a very inhomogeneous way: in some areas the crypts consisted of many cells with numerous mitoses, in others the epithelium was already disorganized.

At this time groups B and C appeared to be in a more advanced phase of recovery.

In control animals, the labelled cells had a typical distribution along the crypt: the frequency was very low in the initial position but increased rapidly to the highest levels in the lower third of the crypt. S-phase cells would then decrease in the median third and were absent in locations higher than 80 per cent of the total height.

Statistically significant differences in the distribution were observed among control animals killed at different times of the day (Becciolini et coll. 1983). These values are illustrated in the figures as broken line curves and reported together with those of irradiated animals killed at the same hour.

The frequency of labelled cells along the crypt clearly indicated the effect of ionizing radiation.

In the different control groups the mean number of cells in the left side of the crypt at different times of the day ranged between 37.5 and 38.9.

Six hours after exposure the frequency of labelled cells along the crypt was markedly reduced in all groups (Fig. 1), being particularly evident in the lower half of the crypts. In group C the decrease
Fig. 3. 20 h after irradiation. Mean distribution frequency of labelled cells (cf. Fig. 1). Mean number of crypt cells was 24, 20, 24 and 22, respectively.

was most marked. The number of crypt cells was also significantly reduced in all groups.

At 12 h after irradiation (Fig. 2) a further decrease was observed in labelled cells; they were mostly localized in the lower part of the crypt, whereas they were absent in the upper 40 per cent. At this time also, the greatest reduction appeared in group C.

Twenty h after irradiation (Fig. 3) a further reduction in the number of cells lining the crypts was found in all groups. Labelled cells reached higher locations compared with previous intervals and their frequency also appeared higher. In group A the cells which incorporated 3H Thymidine reached positions at the villus border.

Extension of the proliferative compartment with labelled cells also at the top of the crypt was a characteristic feature 36 h after irradiation (Fig. 4). In the lower part of the formation the frequency of labelled cells increased towards control values in groups C and D.

Distribution of labelled cells at 48 h (Fig. 5) was similar in all the groups with a significant decrease in frequency at the bottom of the crypt and an equally significant increase in the upper half. It should be mentioned that at this interval and at the next one those crypts were counted which showed a kind of alignment and were not therefore representative of the whole specimen.

Seventy-two h after irradiation (Fig. 6) a lack of homogeneity among the different areas in the sections was remarkable: in some areas the injury was severe, in others recovery had set in. In those areas where counting was possible, diffuse proliferation with homogeneous frequency of S-phase cells along the whole crypt was clearly evidenced in groups A, B and D.

A marked reduction in S-phase cells was still present at the bottom of the proliferative compartment in group A.

In group C, where a more marked recovery in the areas counted led to a number of cells much higher
than in the others, a lower frequency of labelled cells was recorded in the higher part of the crypt.

**Invertase activity.** In the control animals an evident circadian dependence of the invertase activity was found, with a peak at night and a drop in the afternoon. Fig. 7 shows only the values from the second tract, the one corresponding to the tissue sample and in which the activity was close to the highest levels. The remaining tracts showed a similar curve.

Irradiation of epithelial cells at different functional moments led to variations with different curves after irradiation, although the general behaviour was similar to that observed previously in other brush border enzymes. When exposure occurred at maximum enzyme activity (group A) invertase did not reach minimum circadian values (BECCIOLINI et coll. 1974, 1975, 1977, 1979, 1982) but its value increased continuously until 20 h after irradiation. At 36 h it was significantly lower than in controls and continued to decrease until 72 h after exposure, reaching values close to zero.

When the irradiation was given during the reduction phase (group B) the activity increased progressively following the circadian curve. The highest value was higher than the maximum circadian value ($p<0.05$) whereas the subsequent reduction showed the same behaviour as in the preceding group.

When the irradiation was performed at minimum circadian value (group C) the activity paralleled the course in the controls. When the irradiation was given at 6.00 p.m. (group D) no circadian reduction in the activity occurred, and as late as at 36 h, unlike in the other groups, values were similar to those in the controls. Also in this case the values were close to zero during acute injury.

**Discussion**

The present report is part of a research program which aims at identifying the most suitable irradia-
tion conditions which are capable of differentiating the effects in healthy and tumor tissues.

The object of this work was to investigate the behaviour of the proliferative compartment of the intestinal epithelium assessed through the distribution along the crypt of those cells which are still capable of incorporating 3H Thymidine after irradiation. Moreover, since in the upper part of the Lieberkühn crypts the cell differentiating process occurs, recording of the labelled cell distribution also supplies indication of the size of the differentiating compartment.

A block of the proliferative activity was evidenced at the first deaths after irradiation in all groups of animals exposed at different times of the day. At the next intervals some S-phase cells were still present in the lower part of the proliferative compartment and these cells would partly support the epithelial lining.

Thirty-six hours after irradiation the frequency of S-phase cells was high even in the upper part of the crypt. At the same time the enzyme activity decreased.

The high frequency of S-phase cells in the crypts at 48 h and particularly at 72 h demonstrated the remarkable recovery of the intestinal epithelium. At this latter interval S-phase cells were distributed along the whole crypt and in part at the bottom of the villi. The limited extent of the differentiating compartment occurred at the time when the brush border enzyme activity was close to zero.

By comparing the different irradiated groups reasonably homogeneous behaviour was generally observed during the intervals investigated, apart from group C in which the reduction in S-phase cells was more marked than in other groups.

Reduction in the number of cells capable of incorporating labelled nucleoside may have been affected by several factors: (a) the death of proliferative cells in the most radiation sensitive phase of their cycle, demonstrated by the presence of positive Feulgen fragments found in the crypts at early intervals after

Fig. 5. 48 h after irradiation. Mean distribution frequency of labelled cells (cf. Fig. 1). Mean number of crypt cells was 19, 19, 16 and 19, respectively.
Fig. 6. 72 h after irradiation. Mean distribution frequency of labelled cells (cf. Fig. 1). Mean number of crypt cells was 21, 26, 31 and 21, respectively.

Fig. 7. Behaviour of invertase activity in the second tract of the small intestine in control (—) and irradiated (----) animals. The SE limits of the mean values are presented. Groups A, B, C and D, respectively.
irradiation, (b) slowing down of the cell cycle, and (c) arrest in G2 to repair injury before mitosis (RUS-TAD & BURCHILL 1966, DOIDA & OKADA 1969, BACCHETTI & SINCLAIR 1970).

In early differentiating process, already demonstrated in other cell systems (YANG 1974, HARTMUT 1978), could also contribute to reduce the frequency of S-phase cells. Instead of dividing, proliferative cells started synthesizing brush border enzymes which seem to be characteristic for the mature epithelial cells (BECCIOLINI et coll. 1974, 1975). This hypothesis has been confirmed by the increase in the activity of these enzymes at early irradiation intervals. During this period, an increase in free ribosomes or bound to membranes in the epithelial cells of the upper part of the crypts has been observed at electron microscopy (BECCIOLINI et coll. 1976), which could indicate an increase of protein synthesis.

The initial reduction of the frequency of S-phase cells and their marked increase in the whole crypt at 72 h appear important considering that the S-phase lasted for about 60 to 70 per cent of the cell cycle time (CARNIE et coll., AL-DEWACHI et coll.). The results of the initial intervals can be explained by the early differentiation process; while at 72 h, during the acute injury, the lack of brush border enzymes can be explained by the absence of the differentiating compartment.

It is worth noting that no increase in frequency was observed at the bottom of the crypt in which are located G0 stem cells (CHENG & LEBLOND 1974b, LEBLOND & CHENG 1976, POTTEII 1976) and differentiated cells such as Paneth and enteroendocrine cells.

Conclusion. Among the groups irradiated at different times of the day no substantial differences in the distribution of labelled cells were observed during acute radiation induced injury. Modifications in brush border enzymes and in the location of labelled cells led to the hypothesis of an early differentiation process. Differences in behaviour of enzyme activity in irradiated groups were not supported by significant differences in the distribution of labelled cells at corresponding time intervals. On the other hand it should be considered that brush border enzyme synthesis occurs also during migration along the villi (NORDSTROM et coll., JAMES et coll.) and the content of this compartment is quantitatively more important.

SUMMARY

The S-phase cell distribution has been analysed to evaluate the behaviour of proliferative cells in the intestinal epithelium after irradiation at different times of the day. A marked reduction of S cell frequency was observed at early intervals after abdominal irradiation; this reduction was particularly evident in the lower half of the crypts. At subsequent intervals a progressive extension of the proliferative compartment, with labelled cells also at the top of the crypt, was present. The irradiated groups generally showed a homogeneous behaviour even if a more marked reduction in S-phase cells was observed in group C. The invertase activity, a brush border enzyme synthesized during the differentiation process, presented a different behaviour at the early intervals in the irradiated groups. When the extension of the proliferative compartment occurred the invertase activity reached values close to zero. The modifications in brush border enzymes and in S-phase cell distribution, at early killing times, led to the hypothesis of an early differentiation.

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