The effects of irradiation at different times of the day on rat intestinal goblet cells

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Abstract. Quantitative changes in jejunal goblet cells were studied in control and whole body irradiated rats using PAS-Alcian blue staining of crypt sections. A circadian dependence was observed when control animals were killed at different times during the light/dark cycle. Irradiation with 3 Gy produced a 2–3-fold increase within 36 h in goblet cells relative to controls, followed by a reduction to very low levels. There was a return to pre-treatment levels later than was observed for the columnar cells. The present results on the pattern of response of goblet cells and those of brush border enzyme activity are consistent with the hypothesis that ionizing radiation can influence differentiation. In fact during the first hours after irradiation an early induction of differentiation is evident while during the early repopulation phase columnar cells prevailed relative to the goblet cells. Only at later times were normal differentiation patterns seen. Groups of animals exposed to the same dose of radiation at different times of the day showed similar general patterns of behaviour even if the group irradiated at midnight showed a more marked and longer lasting injury.

Goblet cells are quantitatively the most important population of the small intestine epithelium after the columnar cells, amongst which they are intercalated. Goblet cells secrete mucus to provide a lubricant and barrier for the intestinal mucosa. The neutral and acidic mucopolysaccharides synthesized by the cells cover the epithelium and protect the cells from the intestinal micro-flora and toxins and facilitate the passage of the alimentary bolus preventing micro-traumas. The production of mucopolysaccharides begins in the goblet cells of the crypt and continues during goblet cells migration and maturation on to the villus. Goblet cells also participate in the catabolism of iron (Refsum & Shreiner 1980).

According to the unitary theory of epithelial cell origin, goblet cells, and all other types of cells of the small intestinal epithelium, originate from pluripotent stem cells present at the base of the crypt (Leblond & Cheng 1976). Clonal regeneration studies after irradiation and other studies using lectin binding, cell cloning and transgenic approaches provide strong support for this theory (Inoue *et al.* 1988, Potten & Hendry 1983, 1995, Potten 1990, 1995, Potten & Loeffler 1990, Wright 1996).

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During migration in the crypt towards the villus, the goblet cells mature and lose the capacity to divide. In fact, only the oligomucous cells, which are partially differentiated show mucus synthesis and mitosis while they are located in the lowest part of the crypt (Merzel & Leblond 1969, Cheng 1974).

Some cell kinetic parameters such as the total number of goblet cells per crypt, general labelling index, the mitotic index and the distribution of labelled cells along the crypt, have been determined. These parameters exhibit a differential response when the same dose of ionizing radiation is administered at different times of day (Becciolini *et al.* 1982b, 1983b,c, 1996). Several aspects of the behaviour of goblet cells are still not understood.

Therefore, the present study was undertaken in order to clarify the following: (1) whether a variation exists in goblet cell index during a 24 h period, i.e. a circadian rhythm? (2) whether the goblet cell distribution changes after irradiation, and (3) whether irradiation of rats with the same dose at different times of day produces differences in the number of these cells.

MATERIALS AND METHODS

Female Wistar rats 12 to 14 weeks old and weighing 180 to 200 g were used. The animals were kept under standard cage conditions, under a rigid light/dark cycle (lights on 6.30 am to 18.30 pm) with food (Rat Standard Diet, Morini, Reggio Emila, Italy) and water provided *ad libitum*. The polypropylene cages, each containing 3–4 rats, were changed every day early in the morning. Groups of 12–16 animals were used as controls and these were killed at 0.00, 6.00, 12.00 and 18.00 h.

The other animals were divided into four groups and were given a whole body exposure to 3 Gy of γ-rays from a telecobalt unit (dose rate=80 cGy/min) at 0.00, 06.00, 12.00 and 18.00 h. The animals were killed, together with one or two control animals, by ether overdose according to institutional guidelines, in groups of 5–6 from 12 h to 62 days (specifically, 12, 20, 36, 48 and 72 h and 5, 11, 20, 30, 45 and 62 days) after exposure.

In order to estimate the S phase cell distribution in the crypt and the S phase fraction (labelling index) 3.8 MBq of 3H-thymidine (Amersham, Little Calfont, UK, specific activity 74 GBq/mmol) was injected intraperitoneally 1 h before killing. Immediately after sacrifice, the small intestine was removed and treated as in previous studies (Becciolini *et al.* 1976, 1983a,b,c, 1995).

A 1 cm long segment of proximal jejunum from each animal was opened, gently washed in cold saline, and fixed on velcro to keep the tissue rigid during processing and to improve the alignment of the crypt-villus formations. The tissue was fixed into Carnoy's fixative and embedded in polystyrene (Frangioni & Borgioli 1982).

Histological sections 3 μm thick were stained with haematoxylin and eosin and periodic acid Schiff's–Alcian blue-haematoxylin (PAS). The number and position of crypt goblet cells on the left side of at least 50 well-aligned crypt-villus formations were counted in each animal. Only goblet cells with PAS positive cytoplasm together with an evident nucleus were counted. Furthermore, using the number of the epithelial cells along the left side of the crypt, i.e. in the crypt column, the goblet cell index (GCI) was evaluated. The mathematical model of the single cosinor (Halberg *et al.* 1972) was used to evaluate the existence of a circadian dependence in the goblet cell index and other quantitative morphological parameters in the control animals.

The mathematical model of the single cosinor permits the calculation of the temporal distribution of a parameter that oscillates between a maximum and a minimum

with a postulated 24 h periodicity. The mathematical model is represented by $y(t) = M + A \cos 2\pi(t + \phi)$, where $y(t)$ is the observation as a function of hour of the light/dark cycle, M (mesor) is the rhythm adjusted mean, A (amplitude) is the measure of one-half of the extent of the rhythmic cycle and ϕ (acrophase) is a measure of the timing of the lag from a reference point to the peak time of day.

Parameters M, A and ϕ are calculated by linear least squares fit of the cosine model with a 24 h period. The results are reported as a polar representation in which the vector points to a spot (see Figure 1) which represents the mean circadian rhythm, with its length proportional to A and the angle formed with the polar axis proportional to ϕ. The ellipse drown around the tip of this vector covers 95% of the confidence region for the circadian rhythm. The two 95% acrophase confidence limits are found drawing the tangent lines to the ellipse projected from the centre for the 'clock' circle.

The Student's t-test was used to compare the differences between irradiated and control animals. Mean values $\pm$ SE were expressed as a percentage of controls killed at the same time
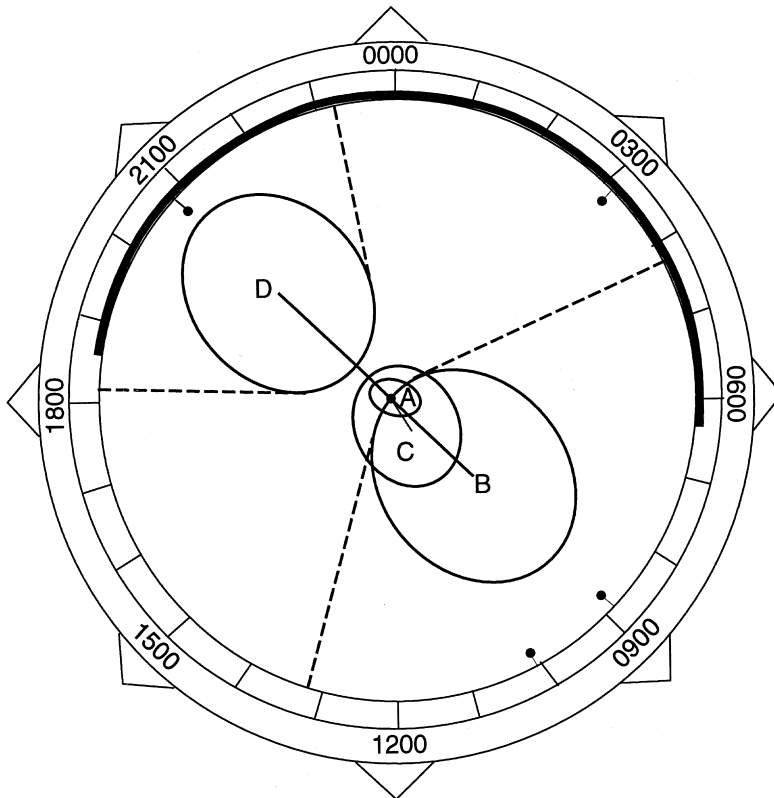

Figure 1. Single cosinor display of total cell number (A), mitotic index (B), labelling index (C), and goblet cell index (D) in the jejunum of different groups of control animals sacrificed at different times of light/dark cycle. The 95% confidence intervals are marked as ellipses. The acrophase, the moment of maximum for each parameter is shown ($\bullet$), and the 95% confidence limits for the acrophase are shown as the dotted lines. The amplitude of each phenomenon is shown by the solid lines.

Table 1. Changes in the number of epithelial cells, goblet cells and goblet cell index in jejunal crypts throughout a 24 h period in control rats

Time of sacrifice (h)	Number of animals	Epithelial cell number (mean + SEM)	Goblet cell number (mean + SEM)	Goblet cell index (%) (mean + SEM)
0.00	12	38.23 + 0.22	1.28 + 0.07	3.37 + 0.19
6.00	14	38.84 + 0.28	0.99 + 0.05	2.56 + 0.14
12.00	15	37.89 + 0.37	1.06 + 0.05	2.78 + 0.12
18.00	16	38.37 + 0.34	1.31 + 0.09	3.41 + 0.22
Total mean		38.33	1.16	3.03

of day. The results for the rats killed at 20 h after irradiation were compared with controls sacrificed at the nearest time point. Statistical significance of the differences is reported in the figures as one asterisk if $P < 0.05$ and as two if $P < 0.01$.

RESULTS

Table 1 demonstrates that, even if the number of epithelial cells in the control animals killed at different times of the day, remained substantially the same during the light/dark period, the goblet cells index showed a cyclic pattern during the 24 h time span with values which increased progressively from 06.00 h to 18.00 h. Approximately 3% of the epithelial cells of the jejunal crypt are PAS positive goblet cells. Analysis by means of the single cosinor method demonstrated a circadian dependence ($P=0.0005$) with a peak at 21.00 h and 95% confidence limits between 18.00 h and 23.00 h (Figure 1). When an ellipse in Figure 1 overlaps the centre of the circle the variations in that parameter during the light/dark cycle are not statistically significant and the differences cannot be attributed to a circadian dependence (D in Figure 1) corresponds to the time at which the crypt labelling and mitotic indices were at a minimum level.

A few hours after irradiation the damage to, and death of, columnar cells are accompanied, in all groups of animals, by a statistically significant increase in goblet cells which appear full of mucus (Figure 2). At 48 and particularly at 72 h goblet cells are less in number and poorly PAS positive in some groups. The behaviour differs among different groups: in the animals exposed at midnight, the early increase in goblet cells is seen only at 12 and 20 h after exposure (Figure 2a). Thereafter, the values are similar to controls with a statistically significant reduction at 3 and 5 days. In rats irradiated at 06.00 h (Figure 2b) the significant early increase persists until 36 h and this is followed by a statistically significant reduction at 48 and 72 h; in rats exposed at midday (Figure 2c) the early increase is present for longer, i.e. up to 48 h. When the animals are irradiated at 18.00 h a progressive increase is observed until 36 h (Figure 2d) when the highest levels of goblet cells in the entire experiment was observed.

In all groups the lowest number of goblet cells in the crypt was observed 72 h after exposure while at 5 days (except for the midnight group), control values were re-established. At later times the goblet cell numbers oscillate around control levels except for the midnight group (Figure 2a) which showed a statistically significant reduction even at 20, 30 and 45 days after exposure.

After irradiation epithelial cells in the crypt were destroyed and there was a block of proliferative activity which continued for as long as 20 h (Becciolini *et al.* 1996). The number of epithelial cells reached a minimum at 20 h and then increased again as a result of repopulation; after temporarily exceeding the control values, the number of epithelial cells returned to normal values.

In order to take into account the simultaneous changes in other components of the epithelium, the variations in goblet cells were expressed as an index. The changes of the total cell population, however, were relatively similar in the four irradiated groups. At the early times after irradiation the values of the goblet cell index (Figure 3) were notably more elevated than those of the number of goblet cells (Figure 2), reaching levels which were as much as three times higher than those of controls in the 18.00 h group (Figure 3d).

However, the general behaviour of the goblet cell index is similar to that of goblet cell number. The maximum value in each group was generally noted between 12 and 20 h after irradiation. However, the highest levels in the 18.00 h group (Figure 3d) were noted at 36 h, at a time when the other groups had already shown a gradual decrease; in particular the midnight group (Figure 3a) had reached control levels at this time. In all cases the minimum values were reached at 72 h, a time of maximum regenerative proliferation in mice (Potten 1990). A successive return to control levels occurred in all groups with the exception of those
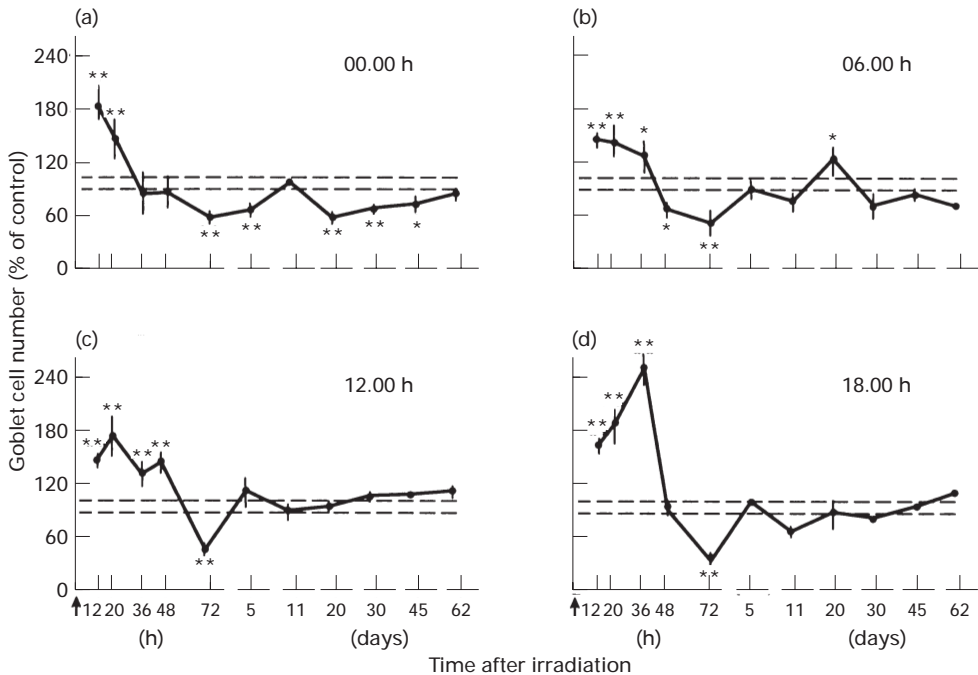

Figure 2. Goblet cell number along the side of the crypt in groups of animals irradiated at four different times of the light/dark cycle: midnight (a), 6.00 (b), midday (c) and 18.00 h (d). The mean values $\pm$ SEM are shown expressed as a percentage of control values from animals sacrificed at the same time of the day. The limits of the mean standard error for the controls are shown as the dashed line. The arrow shows the time of irradiation. The first five time points are measured in hours and the last six in days.

irradiated at midnight which remained clearly below the control levels. In all groups there were some temporary oscillations some of which were statistically significant (Figure 3).

DISCUSSION

The goblet cell frequency in crypt sections might be influenced by: (1) the clonogenic cell number; (2) changes in the differentiation level of goblet cells, e.g. modifications in production and/or secretion of mucus as seen by PAS reactivity; (3) goblet cell death and goblet cell production; (4) changes in crypt shape, e.g. diameter or growth related to (1); and (5) various combinations of these which may vary with time after irradiation.

The results of this study demonstrate, first, the existence of a circadian trend in the number and index of the goblet cells in the crypt and, second, that the maximum number of goblet cells is present at 21.00 h. However, the total number of epithelial cells does not show bioperiodic oscillations (Becciolini *et al.* 1996). The synthesis and accumulation of mucopolysaccharides results in an increased volume of these cells. However, since only those cells with observable complete nuclei were counted, the influence of this phenomenon as a possibility of error must be considered small. Some authors assert that goblet cells undergo several secretory cycles of 12 or 24 h duration during the turnover of epithelium (Neutra & Leblond 1966, Forstner 1978). Another hypothesis which may explain the circadian trend is that there are waves of differentiation with a 24 h periodicity (Becciolini *et al.* 1982a). On the other
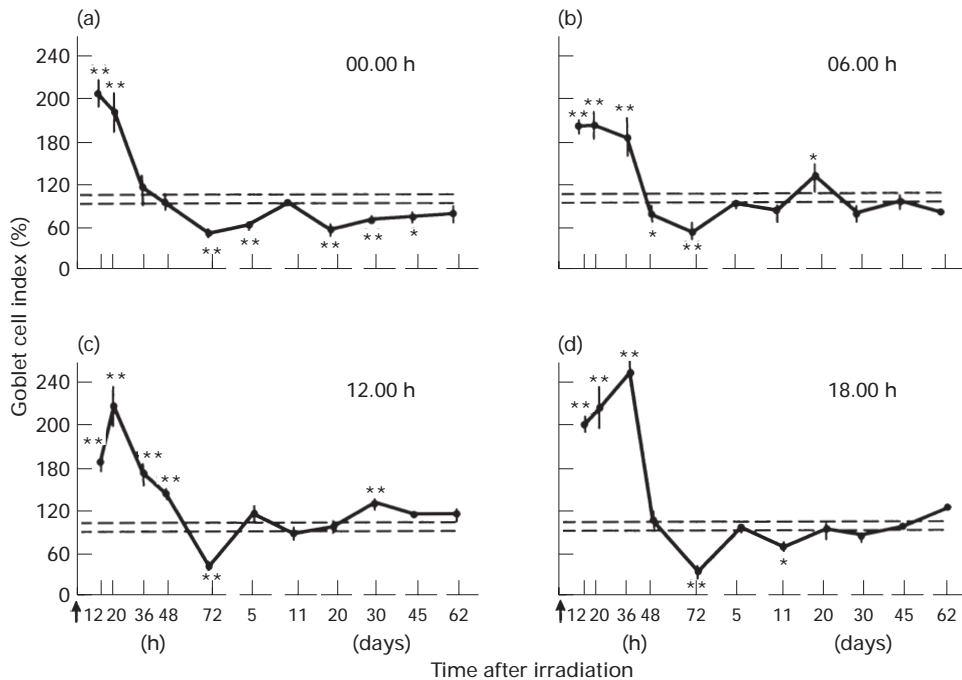

Figure 3. Variations in the goblet cell index (goblet cells to total epithelial cells) as a percentage of controls, in the groups of animals irradiated at four times of the day (see Figure 2 for further details).

hand, the fact that there are very few goblet cells per crypt makes an evaluation of their distribution along the crypt of low significance when seeking to define specific moments of differentiation. It should be noted that at the end of the light period and at midnight, when the rats have increased activity and food intake, goblet cells show a statistically significant increase relative to the values at the end of the dark period and at noon. It is worth noting that during the dark period, when activity and food intake are at their highest there is a statistically significant increase in brush border enzyme activity, e.g. dissacharidases, dipepti- dases and others which participate in the terminal digestion of macromolecules (Becciolini *et al.* 1981, 1987c). Both brush border enzyme activity and goblet cells secretion are different functional aspects, related to terminal digestion and mucosal protection, and both of these increase during the period of food intake. During the light period, when the rats are less active, both parameters appear significantly reduced.

Further evidence of the correlation between differentiation and proliferation can be seen in the distribution of goblet cells along the side of crypt which is opposite to that for DNA synthesizing cells. Goblet cells appear very rarely in the lower 25% of the crypt while the S-phase cells have their highest levels in the lower half of the crypt and are absent in the upper 25% (Becciolini *et al.* 1983a, Becciolini 1987).

After irradiation the response of the goblet cells demonstrates three phases: an initial increase in number, then a reduction and finally a return to normal values when compared with controls. Their morphological appearance also varies with time after irradiation; in the early phase they are notably full of secretory products with the nucleus being flattened at the base of the cell; in the intermediary phase of damage the goblet cells are rare and compressed by neighbouring columnar cells and thus have significant changes in shape and general appearance. Normal morphology is restored after 5 days.

The increase in the number of goblet cells and their index, the latter being influenced by the contemporary reduction in the number of epithelial cells, results from the lower radio- sensitivity of differentiating and differentiated cells. In addition, irradiation seemed to produce an acceleration of differentiation, that is a premature or early differentiation of sublethally damaged proliferative cells that can no longer undergo mitosis (Rijke *et al.* 1975, von Wangenheim & Howard 1978, Paulus *et al.* 1992, von Wangeheim *et al.* 1995). The reduction in goblet cell number was evident even when the epithelium was reconstituted with morphologically normal columnar cells or even when there were more epithelial cell than in controls. These parameters only returned to normal levels after longer intervals.

This is further supported by observations on brush border enzyme activity—another indicator of differentiation. These enzyme molecules are synthesized for the first time in cells in the upper third of the crypt and are characteristic of the differentiation process of the columnar cells. During the first 36 h after irradiation there is a significant increase in these enzyme activities followed by a successive reduction which is contemporary with the maximum damage to the epithelium (Becciolini *et al.* 1976, 1977, 1982a, 1995, Becciolini 1987). As an example of the behaviour of brush border enzymes in the jejunum of the same animals (Becciolini *et al.* 1995, 1996), the variations of leucine-aminopeptidase are shown in Figure 4.

One h after injection of 3H-thymidine at the times studied here the distribution of the labelled cells along the crypt showed a marked expansion of the proliferative, and a consequent reduction of the differentiative, compartment (Becciolini *et al.* 1983c,d, 1996). Previous studies with 8 Gy showed, at the first time intervals, a lower increase of the number of goblet cells per crypt section and the goblet cell index (Becciolini *et al.* 1985). With this higher dose more epithelial cells are killed and at 48 h after irradiation the level of goblet

cells was significantly reduced; at 72–96 h the values of both the number and index of goblet cells were less than 20% of controls. A partial increase followed, but in the case of 8 Gy the intestinal epithelium returned to a qualitatively normal columnar morphology 5–6 days after exposure. However, significantly reduced levels of goblet cells were still evident even at 11 days after exposure.

The results of the present study, as well as those obtained in other studies, demonstrate that the recovery of the intestinal epithelium occurs in two stages: in the first, there is a repopulation chiefly of columnar epithelial cells. The efficiency of this can be seen from the overshoot in the number of columnar cells. In the second stage, which appears only later, there is a complete restoration of the epithelial integrity with a return to normal differentiation patterns. On the whole the morphological and biochemical parameters studied demonstrate the specific effects of ionizing radiations on cellular differentiation as seen by the changes in the activity of the brush order enzymes, the goblet cell number and index, and the distribution of S-phase cells along the crypt. Previous similar quantitative studies undertaken in different parts of the small intestine (duodenum, proximal and distal jejunum and terminal ileum) support these results (Becciolini *et al.* 1976). When higher doses, or fractionated doses are used, the changes are of the same general pattern but may be quantitatively more pronounced (Becciolini 1987, Becciolini *et al.* 1976, 1982c, 1983d, 1984, 1985, 1986, 1987b, 1995). The changes described here were seen in all of the groups of animals irradiated at different times of the day, even if there were differences of the levels and duration of the changes: the maximum increase of goblet cell number and goblet cell
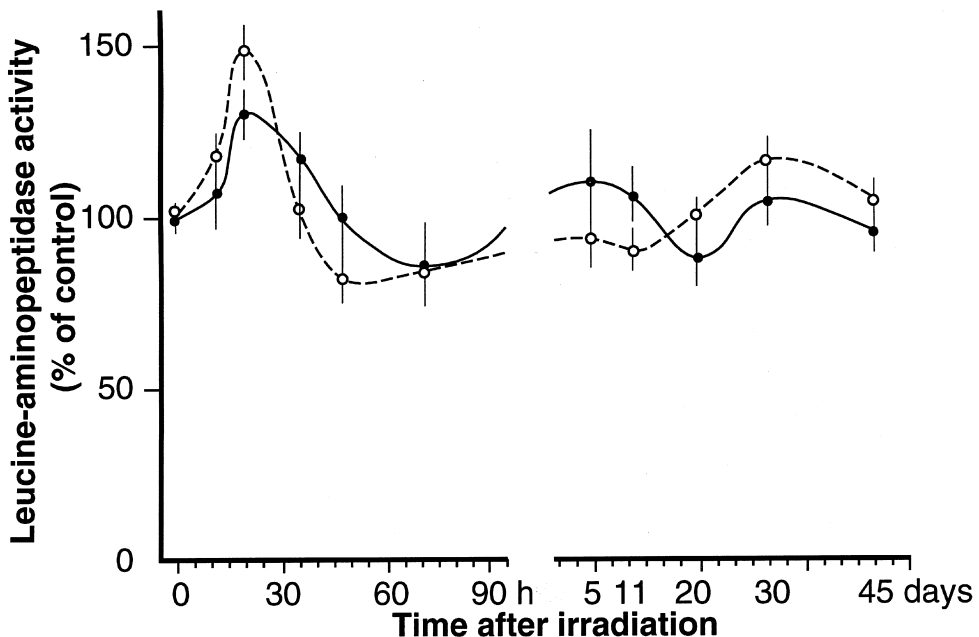

Figure 4. Variations of the leucine-aminopeptidases (LAP) activity in the jejunum of rats irradiated with 3 Gy administered at 6.00 h (solid line) and 18.00 h (dotted line). The activity is expressed as a percentage of control animals sacrificed at the same time of day.

index was in the 18.00 h irradiated group while in the midnight group the duration of the increase was brief and the goblet cells index generally remained significantly lower in relation to the control values for the entire duration of the study.

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