Epithelial cell proliferation and intestinal absorptive function during starvation and refeeding in the rat

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SUMMARY
1. Intestinal epithelial cell production and intestinal absorption were measured in fed, starved and refed rats.
2. Four days' starvation significantly decreased the crypt cell production rate (CCPR), absorption, small intestinal length and crypt cell population.
3. There was an immediate increase in absorption 1 day after refeeding, which preceded a slower increase in CCPR. The absorption rate then decreased progressively after refeeding, and was significantly lower than control levels 1 week after refeeding. The CCPR, however, increased more gradually, reaching control levels after 2 days and then 'overshooting' control values.
4. There was no significant change in the crypt cell population immediately after refeeding; thus we propose that the initial increase in absorption on refeeding is either due to an accelerated maturation rate of the enterocytes or to the migration of enterocytes from the base of the villus to the functional zone.
5. The rapid recruitment of absorptive function appeared to be a 'one-off' event, the villus compartment then having to wait for increased cell production in the crypts to repopulate the villi.

Key words: absorption, cell division, cell migration, food intake, gastrointestinal tract, intestine, proliferation, starvation.

Abbreviation: CCPR, crypt cell production rate.

INTRODUCTION
Intestinal epithelial adaption is a very useful model in which to study epithelial growth control [1]. The control of intestinal epithelial cell proliferation is probably multifactorial, the main factors including the direct and/or indirect effects of food intake (luminal nutrition), local and/or systemic humoral factors and negative feedback from the functional to the reproductive compartments [1]. The study of intestinal epithelial cell proliferation requires the use of robust techniques, capable of accounting for the several changes in proliferative parameters which may occur. One such method is the metaphase arrest technique as applied to microdissected crypts [2-4]. Intestinal function (absorptive capacity) can be measured in vitro by the single-pass segmented-flow technique of Fisher & Gardner [5], which minimizes the problems associated with the susceptibility of the intestine to anoxia and the problems with delayed absorption due to unstirred layers [6-8]. We have recently described a method for the application of both of these methods to the same animal, and have shown that crypt cell production rate (CCPR) is proportional to absorptive function in animals which are in a relative 'steady state' of intestinal renewal and function [9].

The relationship between cell production and intestinal function is of some interest as there is disagreement between those who believe that changes in absorption cannot occur without previous increases in cell production [2] and those who maintain that changes in absorption can occur which are independent of altered cell production [10]. The present study describes the response of these two parameters to starvation and refeeding. Starvation and refeeding is a useful model for the study of intestinal epithelial growth control as it avoids resorting to grossly unphysiological stimuli [11] and can yield valuable information on the hormonal changes associated with altered cell proliferation [12] and on the relationship between crypt and villus cell populations [13]. Moreover, there are profound changes in cell production [13], and the temporal inter-relationship of such changes to the rate of absorption should give insight into the inter-relationship of these two parameters.
METHODS

CCPR

Eight rats were injected with vincristine sulphate (1 mg/kg, intraperitoneally; Eli Lilly, Basingstoke, U.K.) at 09.00 hours. They were killed at timed intervals, within 3 h of injection. Small pieces of intestine from near the ligament of Trietz, or from the mid-colon, were fixed in Carnoy's fluid and the tissue samples were stored in 70% (v/v) ethanol. They were later hydrolysed in 1 mol/l HCl for 6 min and stained with Schiff's reagent. The intestinal crypts were microdissected, the number of arrested metaphases in 20 crypts counted and the mean values plotted against time after injection. The slope of the line, fitted by least squares linear regression, gave the CCPR [4, 14].

Crypt cell population

Tissue was stained with the Feulgen reaction as above. Crypts were individually microdissected and placed on a slide. The length and width of the crypts were then measured with an eyepiece graticule before being squashed. The number of epithelial nuclei in 10 crypts was counted [14].

Intestinal perfusion

The 'segmented-flow' technique for single-pass luminal perfusion of Fisher & Gardner [5] which uses a modified bicarbonate-saline medium with phenol red and 5 g/l glucose was used. The perfusion apparatus consisted of two water-jacketed gut baths mounted on a metal frame, enclosing a water bath for the gassing reservoirs for the perfusion medium and 'saline'. Rats were anaesthetized with ether, the abdomen opened and a catheter tied into the jejunal, just below the ligament of Trietz, and into the ileum (5 cm from the ileo-caecal valve). The intestine was first rinsed with 0.12 mol/l NaCl 0.025 mol/l NaHCO3 solution ('saline') at 38°C. This solution, the medium and the gut baths were all gassed with Medical grade 95% CO2/5% O2. The gut was then perfused alternately with perfusion medium and 'saline'. Rats were anaesthetized with ether, the abdomen opened and a catheter tied into the jejunum, just below the ligament of Trietz, and into the ileum (5 cm from the ileo-caecal valve). The intestine was first rinsed with 0.12 mol/l NaCl 0.025 mol/l NaHCO3 solution ('saline') at 38°C. This solution, the medium and the gut baths were all gassed with Medical grade 95% CO2/5% O2. The gut was then perfused alternately with perfusion medium and gas mix (95% CO2/5% O2). The mesenteric arteries were clamped, the mesentery gently stripped away and the intestine suspended in the gut bath. The fluid that came out of the serosal and mesenteric side of the intestine was collected in a fraction collector. The water absorption rates were determined directly from the weight of fluid secreted during three consecutive 5 min periods (after discarding the first and second fractions).

Experimental design

Six groups of eight rats were used. The first group was a control group (fed normally). The second group was starved for 4 days. The remaining groups were starved for 4 days and refed for 1, 2, 4 or 7 days respectively.

Rats were injected with vincristine at 09.00 hours. Twenty minutes later the first rat was anaesthetized and the small intestine was cannulated. When the gut had been set up for absorption measurement the rat was killed. The time of death was recorded and taken as the time after vincristine injection for the calculation of CCPR. While the first rat gut was being perfused, the second rat was prepared for the second gut bath. The length of the perfused segment was recorded immediately after the gut was removed from the gut bath. The intestine was then dried at 60°C for 2 days or until a constant weight had been reached.

Animals

Male Wistar rats were used (Olac Ltd, Blackthorn, Oxon., U.K.). They were kept in wire-bottomed cages to minimize co-prophagy and were fed a standard laboratory diet (Labshure P.R.D., Christopher Hill, Poole, Dorset, U.K.). They were housed four to a cage. Water was available ad libitum.

Statistics

All results are presented as the mean±SEM for each group of eight rats. Groups were compared by a two tailed t-test for unpaired data. Parameters were correlated by the Pearson product moment correlation method.

RESULTS

The rats lost 23.3±2.3% of their body weight after 4 day's starvation. The mean weight changes for the rats are given in Fig. 1. There was a linear (P<0.001) increase in weight after refeeding. Body weight was restored to the fed level after 3-4 days.

The food intake before fasting was 27.3±1.7 g/day per rat, on the first day after refeeding it was 30.3±2.9 g/day per rat and on subsequent days was 26.9±6.4, 26.7±1.0, 26.3±1.0, 27.5±1.3 and 25.6±0.4 g/day per rat.

Fig. 1 also shows that the CCPR of the duodenum was profoundly decreased by the fast and that upon refeeding it increased gradually. Values near the fed control level were obtained at 2-3 days after refeeding. There was then an overshoot at day 4 (P<0.05). There was no response to refeeding in the colon at day 1, but after this day there was a marked and persistent increase in CCPR.

The effects of starvation and refeeding on total intestinal absorption are shown in Fig. 2. Starvation significantly decreased total absorption (P<0.001). After refeeding there was an immediate increase to near control levels; however, the absorption rate then decreased with time so that absorption was significantly (P<0.05) lower than the control level at day 7. Starvation was also associated with a significant decrease in intestinal length which persisted until day 4 (P<0.01). The decrease in intestinal length means that the increase in absorption per unit length of the intestine was even greater than that of the absorption per whole perfused segment, and exceeded that of the control animals on days 1 and 2. Starvation was also associated with a decrease in intestinal dry weight (from 1.05±0.13 to 0.80±0.14 g). Dry weight decreased further on the first day of refeeding (to
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0.63 ± 0.30 g) and then gradually increased (0.73 ± 0.2 on day 2, 1.09 ± 0.20 on day 4 and 1.15 ± 0.30 g on day 7). The decrease in intestinal weight after starvation meant that the effects of starvation on absorption per mg were minimal, but the initial effects of refeeding were more pronounced (see Fig. 2).

The effect of starvation and 1 day's refeeding on intestinal crypt size and cell population is shown in Fig. 3. There was a highly significant decrease in crypt length, width and cell population after starvation \( (P<0.001) \).

Refeeding caused a small but statistically significant increase in crypt length and width \( (P<0.05) \), but had no effect on cell population size.

DISCUSSION

We have shown that CCPR and absorption are significantly correlated when rats are in a 'steady state' of intestinal cell renewal and function, so that cell produc-
tion will be sufficient to replace cell loss [9]. This was no longer the case in the present study, as absorption changed independently of CCPR.

The weight loss after starvation was similar to that seen in our earlier studies [12]. There was a far greater weight gain after refeeding than that seen in a separate investigation where normally fed rats gained an average of 16.5 g per week per rat over the first 30 weeks of life (R. A. Goodlad, unpublished work). Body weight gain per unit energy intake after refeeding is usually greater than that seen in normally fed animals as starvation-induced energy conservation persists [15]. The refed rats in this study should have reached the unfasted body weight after approximately 10 days of refeeding [15]. There was no sign of post starvation anorexia [15, 16].

CCPR fell markedly after 4 day’s starvation. Refeeding caused a progressive increase in CCPR which returned to near control levels 2 days after refeeding. The CCPR then ‘overshot’ fed levels, with values close to control levels being seen at the last time point studied (7 days). The main changes in proliferative indices observed in starvation and refeeding can be attributed to alterations in the duration of the phases of the cell renewal cycle [17, 18]. Starvation is associated with a slow and gradual decline in labelling index, mitotic index and crypt cell population, while the compensatory changes in proliferation seen after refeeding are more rapid [18]. The increase in absorption noted in the present study peaked before the increase in CCPR, thus the relationship between CCPR and absorption seen in ‘steady state’ models of intestinal adaptation [9] no longer applies when the intestinal epithelium is in a state of flux; in fact there was an inverse relationship between CCPR and absorption in the refed groups ($r = -0.703$). The increase in absorptive capacity after refeeding could be the result of an increase in enterocyte number. We have previously demonstrated that there can be an influx of crypt cells on to the villus within 3 h of refeeding starved mice, and that this precedes increased crypt cell production [13]. Although cell migration was originally attributed to ‘mitotic pressure’ [1], intestinal cell migration without cell proliferation has been observed [19, 20]. Villus cell population can thus be increased by cell migration from the crypts before cell production increases. However, no change in crypt cell population was observed in this experiment. Increases in villus height and cell loss within a few hours of refeeding have been previously described [21]; however, villus height measurements can be confounded by changes in three-dimensional structure [3, 4, 22]. Rat villi are larger than mouse villi and their shape is less regular, thus (in contrast to the mouse studies) it is not practicable to determine cell population by the ideal method of microdissection and direct counting [4]. Stereological techniques [23] could be used to estimate villus volume fraction and surface area, but can only give a relative estimate and must have a stable reference point with which to relate. A newer stereological method measures villus surface area and a ‘shape factor’ and thus is not dependent on a reference point. This method also confirms that while area and shape may be sensitive indicators of the effects of fasting in the rat, estimates of villus height are not [24].

An alternative explanation for the increased absorption seen after refeeding could be that it is the result of increases in the metabolic function of enterocytes, or the result of an increase in the maturation of enterocytes as they migrate up the villus. The expression of absorption in terms of intestinal dry weight also strongly suggests that while the decline in absorption after starvation is due to reduced intestinal mass, the increase after refeeding is not. The absorption measure used in the present study can only indicate the number of functional cells not the total number of villus cells. Only the top third of the villus is thought to be fully functional [25], and therefore function is only an approximate indicator of villus cell population. The subsequent loss of absorptive capacity after day 1 shows that the initial increase was a ‘one-off’ event and that wherever the extra capacity came from it could not be readily replaced.

There are two distinct problems in the quantification of the effects of dietary change (or restriction) on intestinal absorption, namely the choice of an appropriate...
method, finding a suitable manner of normalizing the data [25] and finding a suitable denominator [26]. Absorption per unit of gut is sufficient for a gross description, but, although absorption per cell would be better, one is still left with the problems caused by only a small proportion of all the cells of the villus actually being active in absorption [25]. The study of the functional maturation of enterocytes as they migrate from crypt to villus is now possible [27], and should prove illuminating.

The relationship between villus cell population and crypt cell population is finely regulated, so that there is an almost exact equivalence between the cell population of the two compartments in ‘steady-state’ systems [14]. When the system is perturbed by the chemically induced destruction of crypt cells, crypt cell production is only stimulated once the villus cell population has also been reduced [28], suggesting the presence of a negative feedback mechanism from the villus to the crypts [1]. The results of the present study are not incompatible with such a mechanism, as once the initial, reserve, absorptive capacity of the villi was used, absorption and CCPR were inversely related.

Feedback control can be either from the maturation/functional compartment or from the proliferative compartment [29]. Most of the evidence for negative feedback mechanisms in the control of intestinal epithelial cell proliferation comes from models when tissue damage leads to villus cell population depletion. Although negative feedback may have a role to play in the response to starvation and refeeding, several other factors are also likely to be involved. The presence of food in the gut is one of the most important stimulators of intestinal epithelial cell proliferation [1, 30]. The trophic effects of food intake can either act directly (luminal nutrition or luminal workload) or be mediated via systemic and/or local humoral factors.

In conclusion, we have shown that cell production and absorption are no longer equivalent in the perturbed intestine or refed rats. Absorption increases rapidly after refeeding and then declines as cell production increases, suggesting that there may be a rapid migration of immature enterocytes from the base of the villus to the functional zone or an enhanced maturation of the cells in situ. This rapid mobilization of absorptive function is a ‘one-off’ event and the villi then have to wait for increased cell production from the crypts to refurbish them.

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REFERENCES